



ACTA

SLIPPI

**In Vivo Structure  
of Serum Albumin in  
its Normal Catabolism**

by  
Knut Wallevik

COPENHAGEN 1979



**ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 471**

**From the Finsen Laboratory the Finsen Institute  
Copenhagen Denmark**

**In Vivo Structure and Stability  
of Serum Albumin in Relation to  
its Normal Catabolism**

by  
**Knut Wallevik**

**COPENHAGEN 1979**



Denne afhandling er i forbindelse med omtstående tidligere publicerede afhandlinger af det lægevidenskabelige fakultet ved Københavns Universitet antaget til offentligt at forsvares for den medicinske doktorgrad

København den 6 februar 1979

O Behnke  
dekan

The present thesis is based on the following papers:

- I Wallevik K & H T Mouridsen: The turnover in humans of polymerized human serum albumin and of fractions of human serum albumin with different N-F transformation patterns Prot Biol Fluids 1969 16 755-761
- II Hvidt Aa & K Wallevik: Conformational changes in human serum albumin as revealed by hydrogen-deuterium exchange studies J Biol Chem 1972 247 1530-1535
- III Wallevik K & H T Mouridsen: Detection of metabolic denaturation of serum albumin by gel electrophoresis Scand J Clin Lab Invest 1973 31 225-230
- IV Wallevik K: Reversible denaturation of human serum albumin by pH temperature and guanidine hydrochloride followed by optical rotation J Biol Chem 1973 248 2650-2655
- V Wallevik K: Spontaneous denaturation as a possible initial step in the breakdown of serum albumin in vivo Clin Sci Mol Med 1973 45 665-675
- VI Wallevik K: Isoelectric focusing of bovine serum albumin Influence of binding of carrier ampholytes Biochim Biophys Acta 1973 322 75-87
- VII Wallevik K: 88-interchanged and oxidized isomers of bovine serum albumin separated by isoelectric focusing Biochim Biophys Acta 1976 420 42-56
- VIII Wallevik K: Spontaneous in vivo isomerization of bovine serum albumin as a determinant of its normal catabolism J Clin Invest 1976 57 398-407

These publications are referred to in the text by their Roman numerals



# CONTENTS

	page
INTRODUCTION	7
THE MOLECULAR STRUCTURE AND STABILITY OF SERUM ALBUMIN	10
THE HETEROGENEITY OF SERUM ALBUMIN THE APPEARANCE <u>IN VIVO</u> AND CATABOLISM OF ISOLATED SUBFRACTIONS	15
Dimer and polymer albumin	16
Substances bound to albumin	17
The free SH-group of albumin	18
Intramolecular disulphide interchange in albumin	20
Oxidized albumin	22
Denaturation of albumin during storage	23
Additional albumin heterogeneities with possible relevance for the <u>in vivo</u> catabolism	23
EXPERIMENTAL OBSERVATIONS WITH REGARD TO THE WORKING HYPOTHESIS	24
THE FINAL HYDROLYSIS OF SERUM ALBUMIN AFTER SPONTANEOUS PHYSIOLOGICAL DENATURATION	28
Extracellular hydrolysis	28
Intracellular hydrolysis	29
Endocytosis of serum albumin	29
DISCUSSION OF THE WORKING HYPOTHESIS IN RELATION TO THE ESTABLISHED FACTS ON THE NORMAL CATABOLISM OF SERUM ALBUMIN	33
FURTHER POSSIBILITIES FOR THE CATABOLISM OF SERUM ALBUMIN	35
DISCUSSION OF THE HYPOTHESIS IN RELATION TO THE CATABOLISM OF SERUM ALBUMIN UNDER PATHOLOGICAL CONDITIONS	36
CONCLUSIONS WITH RESPECT TO THE NORMAL CATABOLISM OF SERUM ALBUMIN	40
GENERAL REMARKS CONCERNING PHYSIOLOGICAL POSTSYNTHETIC MODIFICATION OF PROTEINS	40
SUMMARY	42
SUMMARY IN DANISH	43
ACKNOWLEDGEMENTS	44
REFERENCES	45



## Introduction

The motivation for the work which formed the basis for the present survey was the hope of clarifying some of the mechanisms behind the normal in vivo catabolism of serum albumin

The turnover of albumin has been and still is one of the unsolved puzzles of physiology. In broad outline one knows

- 1) that in man about 10 g of albumin is broken down per day (112)
- 2) that the turnover in an organism in steady state<sup>+</sup>) proceeds as a reaction of first order with respect to albumin would have done (129) i.e. the number of albumin molecules degraded per unit time is proportional to the albumin concentration and 3) that the first step in the degradation takes place either within the vascular bed or in a compartment in rapid exchange with it (10 89)

Despite considerable efforts by many groups of scientists it has not been possible to localise the breakdown of albumin to a single organ. The prevailing opinion is that albumin is catabolised in all parts of the body by endocytosis mainly by the endothelial cells of the capillaries followed by proteolytic digestion in the lysosomes (89 47 28 108). Quantitative experiments to support this theory however are still lacking.

The hypothesis for the normal catabolism of albumin which is advanced in this paper was inspired by the work of the late Linderström-Lang - specifically by his picture of a solubilised protein as a molecule in continuous more or less profound structural fluctuation (87). Thanks to an increasing number of

<sup>+</sup>) in medical literature often named as metabolic equilibrium or metabolic balance

experiments which support this idea (55 79 118) Linderstrom-Lang's protein model has gradually won common acceptance. It has been suggested that the structural fluctuations play a fundamental role in the function of protein molecules but they may also imply that the complicated molecules pass through structural conformations which are less stable with regard to denaturation (87). Another important contribution in this field has been Linderstrom-Lang's thesis regarding proteolysis which he had already formulated in 1938 (86) and which is here presented in a slightly modified version: Proteins in their native state are only slowly degradable by proteolysis often even completely resistant to proteolytic enzymes but become susceptible to degradation as a result of partial unfolding; the more unfolded the protein structure the faster the proteolysis. These physical-chemical characteristics for globular proteins form the basis for the following working hypothesis for the normal catabolism of serum albumin (I)<sup>+</sup>. In its native state the albumin is not removed from the extra cellular fluid but during function and the associated changes in the three-dimensional structure the protein becomes susceptible to proteolytic degradation either directly in the blood or after endocytosis.

Experimentally the hypothesis was supported by a well known observation from experiments on the normal catabolism of serum proteins: when an injected tracer protein is denatured it is broken down more rapidly than its native counterpart (39 II III). The enhancement of the rate of degradation of denatured albumin is roughly parallel to the degree of denaturation measured by

<sup>+</sup>) The Roman numerals refer to the author's own work which enters into this paper

physical-chemical methods (39 V) As recently as 10 years ago when the techniques were not so refined as today the measurement of the physiological degradation of a serum protein was usually the only possibility for determining whether it was native or slightly denatured (111 III)

The present paper which is a survey of my own and others' work on albumin falls naturally into two parts:

- 1) an attempt on the basis of investigations made in vitro to sketch the structure and stability of serum albumin under physiological conditions (II IV V VI VII)
- 2) an attempt to clarify whether part of the normal breakdown of albumin in the body could be a consequence of in vivo changes in the structure of the protein (I V VIII)

The present exposition will thus be an effort to combine knowledge from two so different fields as the physical chemistry of proteins and medical physiology

The concepts within the two disciplines do not traditionally have the same value However they are most precisely defined in physical chemistry and those definitions therefore are used in this article This has made it necessary to replace some commonly used medical terms with new ones; for example 'the metabolic stability' of a protein has been termed its sturdiness and the term 'metabolic denaturation' has been replaced by biolability

Unfortunately denaturation a concept widely used in the present survey is not clearly defined I will hold to a relatively new definition proposed by Tanford (132): Denaturation is a major change from the original native structure without alteration of the amino acid sequence i e without severance of any of the primary chemical bonds which join one amino acid to another



The definition implies two obvious weaknesses firstly it calls for a definition of the original native structure ; secondly it includes the imprecise formulation a major change which means that the scope of the definition remains a matter of personal taste In spite of these weaknesses however the definition is the most clear-cut and concise one available A more precise definition of the 'native structure of albumin is part of this thesis and will be set forth later

This survey is restricted to a description of those properties of serum albumin which the author has found relevant for an estimation of its structure stability and behaviour in vivo With regard to other physical-chemical properties of albumin (119 107 108) or more specific problems concerned with the measurement of its catabolism (89 119 107 114 115 113) the reader is referred to the survey articles mentioned

The serum albumin of different mammals has such differences in amino acid sequence (124 107) that it can not be taken for granted that it will behave similarly either in vivo or in vitro However human and bovine albumin which have by far been the most thoroughly investigated do not differ substantially from a physical-chemical point of view Thus a distinction will only be made between albumins when physical-chemical differences have been demonstrated or when data are only available for one species of albumin

#### The molecular structure and stability of serum albumin

An albumin molecule is one single peptide chain of about 580 amino acids the exact number depending on the species The three-dimensional structure is stabilised by 17 disulfide bridges (124) and there is one free NH-group per molecule albumin (53)

The amino acid sequence is almost entirely determined for both bovine and human serum albumin (21 7) The cysteinyl residues are distributed in the amino acid chain in such a manner that the 17 SS-bridges probably form relatively short double loops each including 6 to 43 amino acids (20) The albumin molecule is naturally divided into nine such double loops linked together by a short chain of amino acids (21) The folding of the nine double loops into the three dimensional structure of albumin should thus solely depend on non covalent chemical bonds

The folding of the albumin molecule as if it consists of genuine subunits agrees well with physical-chemical measurements and has actually been predicted solely on the background of such data (IV) Based on observable differences in structural behaviour of the molecule during various considerations the number of subunits has been reduced to 3 or 4 (12 36) which in current terminology are designated as domains (22)

In vitro human serum albumin adjusts to its tightest structure (IV) and the molecule obtains its maximum stability (II) at pH 6-7 and temperatures below 5°C The chemical bonds which keep the above-mentioned domains into their sterical position can be disrupted reversibly by lowering the pH-value to 3.4-3.6 (5) By this NF-transformation<sup>+) (5)</sup> the molecule loses about 10 percent of the native three dimensional structure as measured from its content of  $\alpha$ -helix<sup>++)</sup> (IV 122)

---

<sup>+) N</sup> stands for normal F for fast migration rate in free electrophoresis

<sup>++)</sup> It must be stressed that the  $\alpha$ -helix-content of a protein measured as its capacity to rotate polarised light (optical (Footnote continues at p 12)

The albumin molecule likewise unfolds to a more open and by optical rotation well-defined structure by elevating the pH value from neutral to about 9 (84 49 IV) This loss in structure is also reversible and is called the N-B transition<sup>++</sup>(IV) or the neutral transition (49) The transition from N to B is accompanied by a reduction of the measured  $\alpha$ -helix content of albumin of as little as three percent (IV) As it is probably the side chains of albumin which are involved in the transitions mentioned (84) the overall structural change may be much greater than expressed by the loss in  $\alpha$ -helix At 37.5°C the N-B transition has a midpoint at pH 7.8 (IV) The position of the midpoint and the steepness of the transition is strongly dependent on  $\text{Ca}^{2+}$  ions which in concentrations 10 times as high as the physiological displace the midpoint about 0.6 pH units downwards without influencing the N and B state (49)

At 37°C the albumin is in a temperature-dependent transition between molecular states which are not experimentally well-defined (IV) Probably the molecule has its tightest structure at a temperature below 0°C and starts to denature irreversibly above 50°C before the entire three dimensional structure - determined

---

(Footnote continued)

++) rotation) only gives a fragmentary picture of the complex structure of the molecule However the method is relatively simple and gives one of the few structural parameters we can measure on solubilised proteins The  $\alpha$ -helix content of a protein can not directly be correlated to the stability of the molecule Mutants of a protein e.g. where only one amino acid in the peptide chain is substituted may well have identical optical rotation but still be significantly different with regard to stability against denaturation (142) Sickle cell anemia is a classic example (103 56)

+++ B stands for basic (alkaline) configuration

by weak non-covalent chemical bonds - is lost. At  $37^{\circ}\text{C}$  and pH 7.5 the helix content is 20 percent less than at  $5^{\circ}\text{C}$  (IV)

The pH- and temperature-dependent reversible denaturations take place independent of each other which indicates that different parts (loops or domains) of the albumin molecule are involved in the unfoldings (IV)

Thus at  $37.5^{\circ}\text{C}$  and pH 7.5 serum albumin is involved in relatively steep transitions between different conformations. Consequently small variations in either temperature or pH-value will result in appreciable displacements in the equilibrium constants for the reversible denaturations in question (IV)

The net energy which keeps a protein in its ordered three dimensional structure is surprisingly small at least according to the few cases in which it has been experimentally determined (133); thus the free energy ( $\Delta G^0$ ) change accompanying the unfolding of ribonuclease and lysozyme from their native structure to a restricted random coil (without breaking any SS-bridge) amounts to only 44 and 60  $\text{kJ mol}^{-1}$  respectively (133). As mentioned the unfolding of albumin takes place in several more or less independent steps and for this reason it has not been possible to determine the free energy of complete denaturation of albumin (IV). By hydrogen exchange however it has been possible to get an impression of the amount of the energies which hold the molecule together (II). At pH 6.5 the albumin has its greatest stability but less than 25  $\text{kJ mol}^{-1}$  ( $\Delta G$ -value) is still sufficient to open the molecular structure enough to let the surrounding water enter and come into contact with 60 percent of the peptide bonds of the molecule (II). Increasing the pH-value to 7.6 makes the albumin become more unstable in as much as 70 percent of the peptide bonds are now regularly in contact with the water. These

measurements are carried out at 25°C; increasing the temperature to 35°C causes a further five percent of the molecule a interior to be exposed to the surrounding solution (II) The magnitude of the 25 kJ mol<sup>-1</sup> can be evaluated in relation to the fact that it takes 13-29 kJ mol<sup>-1</sup> to break a single hydrogen bond and that the weakest type of covalent bond (the disulphide bond) requires about 200 kJ mol<sup>-1</sup> (139)

There are appreciable differences in the energies which stabilise the different parts (domains) of the albumin molecules (II-IV) At pH 6.5 and 25°C about 40 percent of the peptide bonds are as mentioned protected against the solvent by  $\Delta G$  values higher than 25 kJ mol<sup>-1</sup> while 35 percent are protected by energies below 17 kJ mol<sup>-1</sup> Among the remaining 25 percent peptide bonds there is an equal distribution of  $\Delta G$  values from 17 to 25 kJ mol<sup>-1</sup>(II)

As mentioned the albumin molecule unfolds from the N to the B state by increasing the pH of the solution from neutral to around nine. When albumin is in the B-state it more easily denatures irreversibly than in the N state. Also the heat-induced loosening of the albumin structure renders the molecule less sturdy against irreversible transformation.

*These conclusions are based on the following observations*  
The rate at which albumin is cleaved by trypsin increases much more by a stepwise change of the temperature from 5 to 40°C and of the pH value from 7 to 8 than can be accounted for only by the increase in activity of the enzyme accompanying the mentioned parameters (137). Furthermore the specific denaturation process which is called intramolecular ES-interchange (which will be discussed in detail later) runs slowly at pH 7 but the reaction

speeds up by increasing the pH value of the solution and reaches a plateau at pH 8.5 (130). The rate of irreversible heat denaturation and denaturation by storage change likewise from a minimal to a maximal level in the same pH interval (138 V).

Beyond this the N-B transition is important for the specific as well as the unspecific binding characteristics of albumin. As an example there is the transition from N to B in the pH interval from 7 to 8 an increase in the binding constants corresponding to an enhanced affinity for the amino acid tryptophan (91) and for  $\text{Ca}^{2+}$  ions (31).

As a last phenomenon I shall mention that binding of different compounds naturally present in the body may influence the albumin structure in a stabilizing manner (see below) analogous to the effect on enzymes frequently seen after binding of substrates.

Thus for the present there is nothing which indicates that albumin in vitro close to physiological conditions should have a structure, stability and mobility which differs substantially from what can be obtained in vivo. Consequently serum albumin must in vivo be in equilibrium between folded and partially unfolded molecules.

The heterogeneity of serum albumin. The appearance in vivo and the catabolism of isolated subfractions.

Industrially produced serum albumin of highest purity appears immunologically homogeneous; nevertheless by physical-chemical methods it can be fractionated into sub-populations and is thus heterogeneous (121 37 VII). Since the invention of the ultracentrifuge it has been known that albumin is heterogeneous and the continuous refinement of physical-chemical methods have

resulted in the characterization of still new classes of heterogeneity. Only in the last decades especially by introduction of refined chromatographic techniques (105 62) and of isoelectric focusing (131 VI VII) has it been relevant to try to discover whether the heterogeneity of albumin occurs in vivo as well (I VIII)

The most important observations and formulations of problems with respect to the heterogeneity of albumin resulted from the work of Joseph Foster and his collaborators at Purdue University Lafayette Indiana (5 8 37 84 99 121 122 123 130 143) but other groups have also contributed notable discoveries. King and Spencer have explored the heterogeneity connected with the free SH-group of albumin (71 72 125) and Fuller-Noel and co-workers were the first to draw attention to the heterogeneity originating from oxidised forms of albumin (41 62)

In the present survey I shall primarily deal with the sub-fractions of albumin which are fairly well characterized and shall especially try to estimate whether corresponding fractions are present in vivo. A survey article on the heterogeneity of albumin has recently been published (61)

#### Dimer and polymer albumin

All preparations of albumin contain 5-15 percent dimer albumin and often a few percent trimer and higher polymers (104). Less than 50 percent of the dimer is formed by intermolecular disulphide bonding (62). The remaining part is dimerized by non-covalent binding forces as it dissociates in a denaturing medium such as one percent sodiumdodecylsulphate under non-reducing conditions.

Purified dimer and trimer human albumin have a turnover rate in man which is respectively 20 and 40 percent higher than for

monomer albumin (1) These rate differences are so small that one should expect to find dimer and trimer albumin in the blood if the polymer species were produced in vivo However it has not been possible to trace polymer albumin in fresh bovine and human plasma (3 1) On the contrary it is found that purified radio-actively labelled dimer albumin is partially converted to monomer in vivo and that the monomerized albumin has the same turnover in man as the native albumin (1)

#### Substances bound to albumin

Serum albumin has the capacity to bind to a large number of naturally occurring substances and drugs Many of these compounds - e g fatty acids (46 26) bilirubin (57) tryptophan (91) and copper (75) - are bound with large affinity to a well-defined section of the albumin molecule i e distinct binding specificity is exhibited Marked advances have been made during recent years with regard to the identification of binding sites on the albumin molecule for different compounds (126 108)

For each of the different substances the binding to albumin results in physical-chemical changes which originate partly from the properties of the bound substances per se (126 VI) and partly from conformational changes induced in the albumin molecule (127 109 27 11) These relatively small molecular fluctuations can depending on the properties of the bound substances be either a loosening of the structure (pharmaca and other unphysiological compounds)(27 11) or a tightening (127 11) The stabilising effect of fatty acids (15) tryptophan (88) and divalent metals (48) can also be reflected in a general protection of albumin against irreversible heat denaturation (15 88) proteolytic degradation (74 76 48) and intramolecular SS-interchange (123



99) When fatty acids are bound to albumin the molecule is to a certain extent protected against unfolding from the N to the F state (122) A similar effect of fatty acids has not been observed on the N-B transition (143)

One of the physiological functions of albumin is to keep free fatty acids in a solubilised form in the plasma (46 16) All industrially prepared albumin preparations contain at least 1-2 molecules of fatty acids per molecule albumin (26) When esterified  $^{14}\text{C}$ -labelled fatty acids are bound to albumin in vitro and the complex injected intravenously the  $^{14}\text{C}$ -activity in plasma is reduced to one percent of the initial value in 10 min At this time the majority of the radioactivity can be recovered from the adipose tissue (16) This indicates that fatty acids bound to albumin are rapidly exchanged between blood and tissue

The alterations in the physical-chemical stability of albumin which result from binding and release of different compounds must also manifest themselves in vivo As a consequence of the rapid exchange in blood of substances with binding affinity to albumin it is difficult to interpret catabolic experiments with albumin made homogeneous with respect to a bound compound However it has been shown that defatted rabbit and bovine albumin are catabolised at the same rate as the nondefatted protein (VIII) As mentioned the pH-value at which albumin unfolds in the N-F transformation is dependent on the number of fatty acids bound to the individual molecules (122) Fractions of human albumin which have different N-F transformation characteristics are not found to differ metabolically (I)

#### The free SH-group of albumin

In industrially prepared albumin the number of free SH-groups

per molecule protein vary from 0.3 to 0.5 and is never larger than 0.8 even with the most meticulous purification methods (71, 3, 35). After a mild reduction with SH-reducing substances such as 0.1 M thioglycolate or mercaptoethanol the value of 1 mol SH per mol albumin is measured (71, 3). The unpaired SH-group is localized to amino acid No. 34 in the peptide chain counted from the amino terminal end (72). The free SH-group of albumin reacts rapidly with low molecular weight SH-compounds at pH values larger than pH 8 under formation of mixed disulphides whereas the same reactions proceed slowly at pH-values below 7 (65, 32). At physiological pH and temperature in vitro the SH-group of albumin is blocked after 20 hours with a 5 molar surplus of cysteine in the solution (32). This is in contrast to the reaction amongst low molecular weight SH-compounds which react quickly at pH-values as low as 6 to 7 (65). These observations led to the supposition that the free SH-group at pH-values below 7 is sterically protected and is exposed to the solvent through the N-B transition (41). Spin labelling indicates that the SH-group in albumin is situated in a 0.95 nm deep pocket (54).

The explanation for the reduced molar ratio of the free SH-group in albumin preparations is either the formation of mixed disulphides by reaction with low molecular weight thiols in serum (71) or oxidation of the SH-group with molecular oxygen to sulphenic acid (41, VII)(see below). The molar ratio decreases during storage which depends further on the storage conditions of the serum from which the albumin is isolated (3). On the background of these findings it has been unclear whether the unpaired SH-group was already partially masked in vivo or whether this happened only after the blood was taken (35). Iodoacetamide reacts quickly with

the free SH-group of albumin already at pH 6.5 (62). By taking blood directly into phosphate buffers 0.1 M pH 6.5 containing different concentrations of  $^{14}\text{C}$ -iodoacetamide with known specific radioactivity, it is determined that the amount of iodoacetamide bound to albumin after it has been isolated from the serum is consistent with a binding ratio of 0.6-0.7 mol iodoacetamide per mol albumin (Vallevik unpublished results). Thus it must be regarded as most probable that the free SH-group of albumin is also partially blocked in vivo.

When the free SH-group of rabbit serum albumin is blocked with  $^{14}\text{C}$ -cystein and this albumin preparation is injected intravenously into a rabbit the cystein will be cleaved from the albumin within 15 min. This means that the free SH-group in vivo is in constant exchange with low molecular weight SH-compounds present in the blood probably in the course of their transportation. It has also been shown that peptides with a free SH-group in vivo can be linked to albumin by means of an intermolecular SS-interchange reaction (61).

#### Intramolecular disulphide interchange in albumin

The seventeen SS-bridges of albumin can by so-called disulphide interchange reactions (SS-interchange) change position within the molecule (3, 123). This process is catalysed either by the albumin molecules own free SH-group (99) or by low molecular weight thiols in the surrounding solution (VII). Bovine serum albumin which has been submitted to such an intramolecular SS-interchange differs from native albumin by having an isoelectric point 0.1 - 0.2 pH units higher (VII) and by a 5.6 percent expansion of the molecule (130, VII). The formation of SS-interchanged albumin is stated to be a reversible process. The reaction rate

measured for the reverse process was considerably slower than the one calculated from the determined equilibrium constant and the rate-constant for the forward process. The reason for this contradiction was as explained by the authors (130) that it had not been experimentally possible to start the reverse process with homogeneous preparations of SS-interchanged albumin. This explanation obviously makes it questionable whether equilibrium actually was obtained between native and SS-interchanged albumin (130)

Purified  $^{125}\text{I}$  labelled SS-interchanged bovine serum albumin is catabolised faster in vivo than is native albumin. Simultaneously it is reversed to albumin with the native isoelectric point (VIII). The albumin which is reversed from the SS-interchanged form has the same physiological disappearance rate as native albumin (VIII)

Intramolecular SS-interchanged albumin is metabolically heterogeneous and the distribution of disappearance rates in purified preparations depend on the circumstances under which the SS-interchanged albumin is produced (VIII). Thus the SS-interchanged albumin which is isolated from industrially manufactured bovine albumin consists of two metabolically distinct fractions. The main part has a half-life of about three days but a not negligible fraction has a half-life of 18 days which is close to the value for native albumin despite the fact that this fraction maintains the isoelectric points of the SS-interchanged isomers (VIII). If on the other hand the SS-interchanged albumin is formed in vitro by catalysis with cystein the metabolic heterogeneity is enhanced and the purified isomers contain a surplus of at least 25 percent albumin which is catabolised with a half-life of less than half a day (VIII)

Ten percent of the albumin in freshly taken bovine serum has isoelectric points identical to the SS-interchanged isomers. A corresponding amount of  $^{125}\text{I}$  albumin purified with respect to the native isoelectric point will after injection adapt to isoelectric points identical to SS-interchanged albumin (VIII). As the in vivo conditions represent a milieu which in vitro promote the SS-interchange reaction (66) it must be considered as probable that albumin in vivo is also converted reversibly to the metabolically more labile SS-interchanged species and that these processes result in a mixture of the two components in the concentration ratio ten to one (VIII).

#### Oxidized albumin

Native bovine serum albumin incubated in vitro in the presence of atmospheric oxygen at conditions otherwise close to physiological is modified irreversibly to albumin with an isoelectric point lower than native albumin (VII). During the process the free SH-groups of albumin are oxidized but no dimer albumin is formed (41, VII). However, it can not be the oxidation of the SH-group which causes the displacement of the isoelectric points as the modified cysteinyl residue can be reduced back to free SH by a mild reduction without simultaneous restoration of the native isoelectric point (VII).

The oxidative modification of bovine serum albumin is localised to the primary structure (VII). Until now however it has not been elucidated which amino acids are involved in the reaction. It might be deamidated amino acids (125) or oxidation of broken SS-bridges to sulphonic acid ( $\text{R-SO}_3\text{H}$ ) (134, 41). Albumin modified by molecular oxygen catabolises 10-30 percent faster than native albumin and does not regenerate in vivo albumin with native

isoelectric point (Vallevik unpublished) It has not been possible to decide whether irreversibly oxidized bovine albumin is present in vivo since the isoelectric focusing by which it is demonstrated itself produces the oxidized form (VII) From isoelectric focusing of normal serum it might however be concluded (VIII) that oxidized bovine albumin with isoelectric points lower than native albumin can not be present in vivo in concentrations higher than a few percent (Vallevik unpublished)

#### Denaturation of albumin during storage

By storage of rabbit serum albumin in vivo as close to physiological conditions as possible it has been demonstrated that albumin denatures The amount of denatured molecules and their degree of denaturation is increased by the incubation time (V 110) The reactions involved in this denaturation have not been clarified but probably both SS-interchange and oxidation are involved After publication of the referenced paper (V) proteolytic activity has been demonstrated in several industrially produced albumins (6) Sodiumdodecylsulphate polyacrylamide gel electrophoresis repeated on the rabbit albumin samples which constituted the material for the observations in reference V and which had been stored at  $-20^{\circ}\text{C}$  for 5 years excluded proteolysis as the possible cause for the demonstrated denaturation (Vallevik unpublished)

Additional albumin heterogeneities with possible relevance for the in vivo catabolism

Chemical modification of a few amino acids in the peptide chain of albumin may possibly take place in vivo Oxidation of albumin with molecular oxygen is an example of such a possibility

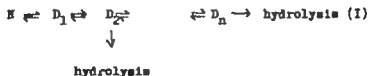
(VII) It has been demonstrated that acetyl salicylic acid in pharmacological doses in vivo acetylates the amino acid lysin in albumin (50) and especially is lysin No 119 counted from the amino end of the peptide chain exposed to this modification (136) This type of reaction may possibly take place in the body between different metabolites and albumin

Finally it is indicated that a fraction of bovine albumin has arginine added to the  $NH_2$ -terminal end - a chemical modification which can only be envisaged as taking place in vivo either during protein synthesis or postsynthetically through enzymatical addition (83)

Albumin with physiologically modified amino acids has not yet been purified from plasma and consequently it is difficult to predict any possible influence on the function or catabolism of serum albumin However it can be mentioned that rabbit albumin or human albumin acetylated with one mol acetyl salicylic acid per mol albumin can not be distinguished metabolically during the first week after injection in rabbits (Vadstrup and Wallevik unpublished results)

Experimental observations with regard to the working hypothesis

The hypothesis for the physiological degradation of serum albumin was described in the introduction and can be formalised in the following reaction scheme (I V VIII):



Reaction scheme I expresses that the native conformation<sup>+) in vivo</sup> can be converted reversibly into different denatured forms  $D_1$   $D_2$   $D_n$  of albumin. According to the theory N should only be catalysed after preceding transformation to one of the denatured forms.

In the previous section it was shown as probable that serum albumin in vivo does not maintain its tightest structure but is involved in various reversible processes which imply transitions to more open and less sturdy conformations. These openings of the structure are provoked by changes in temperature and hydrogen ion concentration and by presence of various ligands. It was further shown that albumin in vivo is converted reversibly into different probably intramolecular SS-interchanged structures. The SS-interchanged albumin probably has the B-form of the mentioned N-B transformation as a basis (99-130).

This information can be formalised in the reaction scheme:



where B is the basic configuration and  $SS_1$   $SS_2$  and  $SS_n$  represent different degrees of SS-interchange. The brackets around the reverse processes imply that both in vitro and in vivo it has been demonstrated that not all SS-interchanged albumin is reversed to native protein (130-VII).

<sup>+) The recognition that albumin is also heterogeneous in vivo makes a re-evaluation of the concept native albumin necessary. In the following discussion I will keep to the physical-chemical definition when talking about physiological conditions and consider native albumin as the configuration of the molecule which under the in vivo conditions is most resistant to denaturation (132).</sup>



Reaction scheme II contains only the experimental findings with respect to the SS-interchange in vitro and must be greatly simplified compared to the in vivo situation. In vivo for example the temperature- and the ligand-induced conformational changes must enter into the chain of processes in an unknown order

The rate constants of the isolated processes in reaction scheme II must be very different from each other as the processes in the N-B transition are fast reacting ( $t/2 < 1 \text{ sec}$ ) (116) while the SS-interchange reactions are slow processes (hours to days) (130 VII)

The fundamental agreement between reaction scheme I and II is evident

With regard to the second part of the hypothesis - that the denatured forms of albumin in reaction schemes I and II should be less resistant than native albumin to in vivo degradation we have the following experimental basis:

- 1) as mentioned it has been shown in vitro that the B-form and the temperature unfolded subspecies are more susceptible to proteolytic degradation than native albumin (137)
- 2) The most important observation in this connection however is that SS-interchanged albumin in vivo is catabolised faster than native albumin (VIII)

From the parameters determined by the turnover of radioactive labelled SS-interchanged albumin in cows the amount of native albumin which might be catabolised through a possible preceding SS-interchange reaction might be quantitatively estimated (VIII)

Ten percent of the albumin has in vivo isoelectric points corresponding to SS-interchanged albumin. If this amount is catabolised with the same half-life of three days as determined for a similar

fraction isolated from industrially manufactured albumin about half of the normally catabolised albumin will have to pass through an SS-interchange reaction beforehand (VIII)

As mentioned above a considerable amount of the SS-interchanged albumin isomer formed by catalyses with cystein is catabolised with a half-life of less than half a day. If only two percent of the albumin in the organism catabolises this fast the degradation of this fraction would account for about 50 percent of the total albumin turnover (VIII)

The cysteine catalysed albumin isomer has not as such been demonstrated in the blood. Nevertheless the estimation just mentioned is included in this paper to draw attention to how small the amounts of fast degraded albumin are which need be present in the blood as part of a reaction chain of type I, to account for appreciable fractions of the total albumin turnover.

It will be difficult to directly demonstrate such small amounts of a modified albumin in the blood. However, if an isomer after parenteral administration can regenerate native albumin this will be a fair indication that the isomer might be propagated in vivo. When the renaturation takes place in vivo the denaturation process must be practicable as well.

As mentioned previously the particular cysteine-catalysed albumin isomer does actually renature after injection (VIII). This, however, together with the fact that the physiological milieu is compatible with the SS-interchange reaction (66) is so far the only indication that the fast degraded SS-interchanged isomers could be physiological products.

The final hydrolyses of serum albumin after spontaneous physiological denaturation

The proposed model for the normal degradation of albumin has up to now dealt with the initial steps in reaction scheme I. A further requirement for the relevance of the theory is that the denatured albumin molecules ultimately be hydrolyzed by the mechanisms normally applied by the organism to degrade denatured proteins (I V VIII)

According to the thesis of Linderstrom-Lang there should be no problems in sorting native protein molecules from denatured when only proteolytic enzymes are present (86)

Before a closer discussion of the final possibilities of hydrolysis it should be mentioned that from turnover experiments with labelled denatured albumins (39 III) two degrees of biolability might be distinguished

- 1) Totally biolabile albumin where the molecule before the injection is transformed to such an extent that it is cleared from the blood in a few minutes
- 2) Partially biolabile albumin where the rate of degradation of the molecule is moderately enhanced compared to native albumin but the denaturation is so minor that it frequently can not be measured by common physical-chemical methods

Extracellular hydrolysis: Proteolytic activity in the blood is slight because of an excess of protease inhibitors (82). However proteolytic enzymes associated with blood coagulation (e.g. plasmin) function extracellularly and proteolytic activity can be measured directly in fresh plasma (6). Protease and esterase activity are as previously mentioned also recognized in even

highly purified albumin preparations an activity which also in vivo could possibly be in close contact with albumin (6) If proteolysis occurs in the extracellular space per se however it is experimentally difficult to trace the resultant peptides as they probably will be rapidly eliminated from the circulating blood

Intracellular hydrolysis: The second possibility - which on the other hand has good experimental support - is that the denatured albumin is picked up by means of endocytosis into different cell-systems of the organism and hydrolysed after fusion of the endocytic vesicles with the proteolytically active cytoplasmic lysosomes (93 47 30 28) As endocytosis of native albumin especially in the endothelial cells is considered to play a crucial role in other relevant theories on normal albumin catabolism as well (108 51 17 18) the problems connected with physiological endocytosis of albumin justify a closer discussion

#### Endocytosis of serum albumin

The process of endocytosis involves the transport into cells of extracellular media by means of invagination of the surface membrane to form a vesicle which then enters the cell The transport of material by endocytosis consequently has two vehicles: 1) the cell surface with its specific adsorptive capacity (adsorptive endocytosis) 2) the extracellular solution which is passively surrounded by the released piece of cell membrane (non-specific liquid endocytosis) (9 58 1 25)

The classical term phagocytosis indicates uptake of particulate material into cells and is mainly restricted to the reticulo-endothelial system In its original designation pinocytosis implies

uptake of solutes. However, there is a continuous spectrum in the size of the ingested material, from small molecules through macromolecules, aggregates to bacteria, hence the common term endocytosis is now often used to cover these varied processes (58, 25, 1). However, there are considerable quantitative differences between the two processes as well as differences in specificity among various cell systems (25, 1).

When totally biolabile albumin is injected intravenously it is picked up by the Kupffer cells of the liver (8) with a half-life of 6.5 min (102). It has no influence on the uptake whether the totally biolabile albumin is monomer or polymer (23) and the uptake is probably not specific for biolabile albumin but for denatured albumin as a whole (100).

If the albumin in reaction scheme I attains a degree of denaturation matching that of totally biolabile albumin, it should also be taken up by endocytosis in the Kupffer cells. As only 10 percent of the normal catabolism of albumin takes place in the liver (51) it is not possible to account for the final hydrolysis of albumin by endocytosis in this organ alone.

However, endocytosis is beginning to be recognised as a common cellular mechanism of transportation in eukaryotes (9, 101, 52) and it has been demonstrated that albumin in vitro is taken up in a variety of cells: endothelial cells<sup>+</sup>) adipose cells (29), fibroblasts (128), liver cells (47, 93, 30), kidney renal tubules (14), wandering phagocytes (13, 33, 94, 73), rat yolk-sac epithelial cells (140, 141) and cancer cells (117, 92, 106).

In most of these investigations the primary interest has been the cellular mechanisms involved in the process of endocytosis.

<sup>+</sup>) Joyner, W. L. and Moriarty, G. M. personal communication.

For technical reasons the albumin used has been labelled with iodine to such a specific radioactivity that it can be stated with certainty that if the labelled albumins had been used in catabolic studies in whole animals they would have turned out to be biolabile<sup>+</sup>) (39). In the few investigations where the authors were aware of the possible influence of the albumin structure on endocytosis it has been shown that native albumin is not taken up by migrating phagocytes (13-73) or amoebae (24) in contrast to denatured albumin which is rapidly internalized. In liver cells (92) and epithelium from the rat yolk-sac (95-96) a positive correlation is furthermore demonstrated between the degree of denaturation of the tracer albumin and the rate of endocytosis. This increase in the rate of endocytosis parallel to the degree of denaturation is due to an increased adsorption of the modified albumin to the cell surface and not to an increased formation of endocytic vesicles (95-96, 141).

If these observations can be transferred to other cell systems a hypothesis for the endocytosis of extracellular proteins can be advanced which follows the same principles as the thesis of Linderström-Lang for the proteolytic degradation (86): native proteins are not internalized in cells by specific endocytosis; however when denatured they are taken up specifically by endocytosis at a rate which is positively dependant on the degree of denaturation.

The molecular mechanisms involved in the selection of the denatured molecules by the cell-surface-complex can be explained

---

<sup>+</sup>) The biolability is a result both of an excessive degree of iodination (i.e. number of iodine atoms per molecule albumin) and a high dose of irradiation from the bound radioactive iodine.

without difficulty on the basis of common knowledge of protein denaturation and protein-protein interaction (133 63) For example in binding of protein substrates to the active centre of proteolytic enzymes the binding affinity is proportional to the length of the peptide chain bound (64) A biolabilisation or a denaturation of albumin will be associated with a partial unfolding of the molecule which means that a larger number of amino-acid side chains are exposed to the solvent and become accessible to binding to the surface of the membrane most likely mainly by hydrophobic interaction (64 63)

If endocytosis is involved in the normal catabolism of albumin it must be the degree of denaturation and not merely an aberrant albumin structure which determines the rate of endocytosis This conclusion can be derived from the experimental finding that human albumin as long as it is native is not catabolised faster in, for example rabbits (39) and dogs (44) than the homologous albumins - although the structure of the human albumin deviates so much from the homologous that the immune system of the animal is activated This sensitisation of course has the consequence that after a week of normal catabolism of the human albumin it is rapidly eliminated from the animal (39)

In the theories based on endocytosis as an important element in the normal turnover of albumin endocytosis is mainly considered unspecific (106 89 119) The consequence of such an unspecific liquid endocytosis would be that native and partially biolabile albumin are internalised at the same rate In order to explain the small differences in degradation rate which are frequently seen amongst albumin preparations it has thus been necessary to postulate a considerable flow of albumin through the cells in

the process of which the denatured molecules should be more quickly hydrolysed in the lysosomes than the native albumin (108 28) The theory presupposes that the vesicles which come in contact with the lysosomes are again released and exocytosed a sequence of events which has not thus far been demonstrated experimentally (59)

The migration of endocytic vesicles and the transport of denatured protein through the endothelial cells of the capillaries have been demonstrated by electromicroscopy (120) On the other hand it has been argued convincingly that this mechanism of transfer can not quantitatively play any major role in the exchange of albumin between the intra- and extracellular space (80)

Adsorptive endocytosis where the affinity of the cell-surface-complex for albumin - and consequently the rate of endocytosis - depends on the protein structure seems thus to be the most likely mechanism for sorting denatured albumin from native prior to the final intracellular hydrolysis

The present potential of purifying well-defined modified albumin (VII) together with the possibility of growing endothelial cells in vitro (60) and determining their uptake of tracer proteins<sup>+</sup>) should make it possible to verify whether the correlation between albumin structure and rate of endocytosis is also valid for endothelial cells and furthermore to decide whether native albumin on the whole is endocytosed by this cell system in vitro

Discussion of the working hypothesis in relation to the established facts on the normal catabolism of serum albumin

No points in the proposed theory on the degradation of

---

<sup>+</sup>) Joyner W L and Moriarty G M personal communication



albumin are in conflict with our present knowledge of catabolism of albumin. As mentioned in the introduction the degradation of native albumin in a body in steady state proceeds as a reaction of first order with respect to albumin (129). Protein denaturation usually follows first order kinetics (85-78); this is also the case for the SS-interchange reaction in albumin catalysed by its own free SH-group (130).

It has been demonstrated that native albumin accumulates in wound tissue. Degradation of the trapped albumin does not take place before it has returned to the normal blood circulation (97). When returned to the circulation the turnover of albumin which has been deposited in a wound is normal (98). These findings support that 1) albumin is only catabolised when participating in the normal exchange between the intra- and extravascular space and 2) a tissue with marked phagocytic activity (macrophages, leucocytes) is not especially catabolically active towards native albumin.

McFarlane, who is one of the pioneers in the exploration of the normal turnover of serum proteins (89), has recently measured the catabolism in rabbits the first 24 hours after injection of radiiodinated albumin and demonstrated that radioactive iodine during the first hours is apparently released from the tracer albumin with only half the rate of that obtained after 24 hours (90). From this data McFarlane and collaborators conclude that an albumin pool somewhere in the animal has to be in balance with the labelled albumin before it is degraded at the maximum rate (90). In the discussion on where in the body this pool may be localised McFarlane assumes that all the albumin is catabolised by a single mechanism. The experimental results he obtained however could just as well be explained by assuming that albumin is

degraded by various mechanisms which may degrade albumin at different rates and until the system has reached a steady state may be recognized as displaced in their release of radioactive iodine to the iodine pool of the body. Experimental observations do exist which support the suggestion that the catabolism of albumin could take place via different mechanisms (119)

Further possibilities for the catabolism of serum albumin

Albumin may escape from the organism by filtration or secretion both to the internal and external surfaces of the body (for survey see ref 119) However within normal physiological conditions only the loss of albumin into the gastrointestinal tract might play any quantitative role

In 1960 it was estimated that about 50 percent of the turnover of albumin in a dog could be explained by exudation (leaking out) of serum proteins into the gastrointestinal tract (44 45) and since then this mechanism for the degradation of albumin has been under discussion. The original investigations have been criticised for the surgical injury considered to give rise to an unphysiological leakage of serum proteins into the intestinal lumen. The substance of this criticism was apparently confirmed by later measurements on the turnover of chromium labelled albumin in healthy individuals (70 135) In these experiments it was found that less than five percent of the normal albumin degradation could take place in the intestinal tract. The basis for the experiments was that free chromium is not absorbed from the intestine which means that the amount of chromium in the feces after intravenous injection of chromium-labelled albumin should be a direct measure of the amount of chromium-labelled albumin which

has escaped to the intestine. However, there are substantial sources of error in the experiments: the labelling with chromium per se denatures the albumin (135-115) and it is not known whether chromium even when it is bound to albumin can be excreted into the intestinal tract in healthy individuals, especially when it is known that the free chromium can not be resorbed.

Leakage of serum albumin into the gastrointestinal tract as a process of quantitative importance in physiology, however, has recently been reinvented on the basis of gentle and careful measurements through a gastroscope and of introduction of soft probes orally into the intestines of healthy persons (19). From these experiments it was again calculated that about 50 percent of the breakdown of albumin proceeds by proteolysis of the protein in the gastrointestinal tract (19). If the remaining part is catabolized by, for example, a spontaneous denaturation and hydrolysis in the vascular bed, it may be presupposed that there might be a displacement in time before the tracer iodine, released by the degradations of albumin, is balanced with the total body pool of iodine.

Other examined sites for the catabolism of albumin are liver and kidney. By perfusion experiments on these organs it has been estimated that less than 10 percent of the total albumin degradation has its origin in each of these organs (51-68).

Discussion of the hypothesis in relation to the catabolism of serum albumin under pathological conditions

Generally speaking, conditions known to increase the degradation rate of albumin in the body (36-114-115-107) and which can not be explained by mechanical loss of albumin will imply an increased probability for protein denaturation as well.

At plasma concentrations of albumin which vary 10 g per liter around the normal value it is found that as is characteristic for first order reaction kinetics the amount of albumin catabolised per unit time depends only on the concentration of albumin in the plasma (119 43). Nevertheless at very low plasma concentrations of albumin a smaller and at artificially increased concentrations a larger first order rate constant has been obtained (2) indicating that the degradation of albumin at extreme albumin concentrations is regulated (18 43 67).

As the basis for the discussion of a possibly regulated degradation of albumin I have selected analbuminaemia an extremely rare (12 cases reported in the literature) but reasonably well examined genetical abnormality (40 69 43). The concentration of serum albumin among these individuals varies from 0.2 to 0.4 g per liter in contrast to the normal 40 g per liter (43). The cause is a defect in the synthesis of albumin as the rate of degradation in these persons in their individual steady states is 3 to 7 times slower than in normal individuals. When the albumin concentration in these individuals is increased to a normal level by infusions the slow rate of degradation is maintained in some of the cases (40 43) while in others the rate is increased to values characteristic for normal persons (69).

These observations are not directly compatible with albumin degradation as a simple first order reaction which would imply that the relative rate of degradation should be independent of the albumin concentration. On this background a theory for normal albumin degradation has been put forward which is in line with the established concentration-dependent degradation of IgG (17 18 67). Briefly the theory holds that degradation and transport of serum protein over various tissue barriers has a common basis (18 67 43).

However in contradiction to the theory based on nonspecific liquid endocytosis (see previous section) specific receptors for the various serum proteins should be available at the cell surface (18 67) The number of these receptors should be limited Proteins bound to the receptors should be transported intact through the cells during the endocytic process while all the proteins which passively accompany (nonspecific liquid endocytosis) should be susceptible to the proteolytic activity of the cell and thus be degraded (18)

At such low plasma concentrations of albumin as are found with analbuminaemia the receptors on the cells should not be saturated and consequently most albumin should be transported intact through the cells while a considerable part of the albumin in normal individuals should be unbound and thus be degraded In the case of IgG such IgG-membrane complexes have been demonstrated in homogenates of the intestines from neonatal rats (67) and they might be present in other cell membranes in adult mice (67) but such complexes are not found for albumin or other serum proteins either in the investigations mentioned or in any other investigations (42)

If the hypothesis of a concentration-dependent degradation of albumin should be brought in agreement with the previously mentioned observations 1) that cells have a special affinity for denatured albumin and 2) that denatured albumin in vivo always is degraded more rapidly than the native protein it will be necessary to postulate additional cellular mechanisms for sorting Either a) only the native albumin is protected by the coupling to the receptors while the denatured albumin is degraded although it is membranebound or b) specific receptors exist for both native and denatured proteins in such a manner that these can be processed differently by the cell

However none of the hypotheses so far advanced for the normal degradation of albumin explain why the turnover in some individuals with analbuminaemia remains slow even after the albumin concentration in serum is artificially brought to the vicinity of the normal (43)

A mechanism for the degradation such as that sketched in reaction scheme I however involves several reversible processes where changes in the concentrations of the single components would be able to regulate the overall process. As an example the slow disappearance rate of albumin in albuminaemic persons in their habitual steady state could be explained by the high concentration of free fatty acids in plasma also observed in these individuals (69). The high plasma levels of free fatty acids could displace the concentrations in the reaction:  $\text{albumin} + \text{free fatty acids} \rightleftharpoons \text{complex (albumin-fatty acids)}$  in favour of the bound form of albumin. This form is as mentioned earlier partially protected from denaturation and might consequently also have a slower catabolism.

However it must be stressed that analbuminaemia is a disorder which might mobilise rather special mechanisms in the individuals concerned which implies that it is questionable to extrapolate to normal conditions. The few cases reported could also have different genetical backgrounds and thus a different etiology.

Not only are the experimental facts about the physiological degradation of serum albumin sparse but different investigations of the same problem have - as would have appeared - often given conflicting results. The current discussion on the degradation of serum proteins is, as illustrated, encumbered with many unknown factors. These again make it necessary to employ many assumptions which might be difficult or impossible to substantiate. In this respect

the work presented here and the discussion of its results are no exceptions. This presentation has dealt with a fragment which I have examined in relation to the whole. Only additional experiments might disclose whether the proposed location of the result within the normal catabolism of albumin is correct.

Conclusions with respect to the normal catabolism of serum albumin

The main objective of the preceding exposition has been to point out that denaturation of serum albumin can take place in vivo as a consequence of the structure and stability of the molecule and to emphasize this mechanism as a probable and in case quantitatively important factor in the normal degradation of albumin.

The total loss of albumin by the mechanisms described in the literature is estimated to account for around 50 percent of the total catabolism (68). If an additional 50 percent is degraded through the physiological denaturation rendered probable in the present paper, the main part and possibly all of the normal albumin degradation should be accounted for. Together with the probable loss of albumin into the gastrointestinal tract, the in vivo denaturation described completes the picture of the albumin catabolism as a result of an inevitable [unspecific] randomized loss of albumin. If that is the case, the only factor common to these two mechanisms is that albumin ultimately is hydrolysed by the proteolytic enzymes of the body in such a way that the amino acids are recycled.

General remarks concerning physiological postsynthetic modification of proteins

The primary structure of a protein determines its three-dimensional folding and thus its function (4). The consequence of the observations presented could be that the primary structure by

deciding the stability of a protein at the same time determines its lifespan in the body

The comparisons made between the stability of albumin in vitro and in vivo have often been criticised on the basis of the assumption that any incubation in vitro under conditions close to the physiological involves a heavier strain on the protein than is so in the physiological state. An essential result of the investigations reviewed is the indication that even a molecule as sturdy as serum albumin has neither its most compact structure nor its optimal stability under in vivo conditions. Therefore returning once more to Linderström-Lang (87) we generally have to reconsider the commonly accepted but never proven concept of physiological conditions as especially stabilizing for proteins. It is probably nearer the truth that a functioning protein is a population of molecules with small differences in structure and stability but in equilibrium with each other. The equilibria are to a certain extent influenced by the environment which means that the distribution among the different species of molecules is displaced by changes in the physiological conditions. The resultant molecular fluctuation or breathing is fundamental for the many-sided functions of proteins but also has as a consequence that the proteins might pass through molecular conformations which are vulnerable to irreversible denaturation or proteolytic degradation.



### Summary

From the literature and from the author's own investigations it is illustrated that the albumin molecule in vivo has neither its tightest structure nor its maximum stability against denaturation but has a flexible structure and participates in transitions between different conformational states which are determined by the hydrogen ion concentration, the temperature and substances bound to the molecule. The opening of the albumin structure in question results in an increased probability for intramolecular SS-interchange and other types of denaturation.

It is demonstrated that albumin in vivo may be converted to its SS-interchanged isomer and that SS-interchanged albumin can be reversed to native albumin. This has the consequence that 1/10 of the albumin in vivo is present in the SS-interchanged form. The isomers are catabolised considerably faster than native albumin. Assuming that the SS-interchange reaction is part of the normal catabolism of albumin, it is estimated that at least half of the albumin passes through this isomerization before it is broken down.

In connection with other statements in the literature on the turnover of albumin, the conclusion is drawn that it is not necessary to presuppose specific mechanisms to handle the degradation of albumin. The main part of the catabolism can be explained by an inevitable randomised loss of albumin by denaturation where the final elimination of the denatured albumin takes place by unspecific proteolysis either directly in the extracellular space or in the cytoplasmic lysosomes after preceding endocytosis. As an additional mechanism for elimination demonstrated by others, albumin is continuously exuded from the body especially through the epithelium of the gastrointestinal tract.

## Summary in Danish

Ud fra litteraturen og egne undersøgelser er det sandsynliggjort at albuminmolekylet in vivo ikke besidder sin tætteste struktur eller har sin maksimale stabilitet over for denaturering men har en fleksibel struktur og befinder sig på overgange mellem flere forskellige strukturelle tilstande der betinges af hydrogen-jon koncentration temperatur og substanser som kan bindes til molekylet. De involverede åbninger af albuminstrukturen medfører en øget sandsynlighed for intramolekylær SS-oggruppering og anden form for denaturering.

Det er vist at albumin in vivo omdannes til dets SS-oggrupperede isomerer og at SS-oggrupperet albumin kan tilbagedannes til nativt albumin. Ca. 1/10 af albuminet er in vivo til stede i SS-oggrupperet form. Disse isomerer kataboliseres betydeligt hurtigere end nativt albumin. Ved at antage at SS-oggrupperingen er et led i den normale katabolisme af albumin er det skønnet at mindst halvdelen af albuminet passerer gennem denne isomerisation før det bliver nedbrudt.

Sammenholdt med de øvrige angivelser i litteraturen om albumins omsetning kan det konkluderes at det ikke er nødvendigt at forudsætte specifikke mekanismer til nedbrydning af albumin. Stereotyperne af katabolismen kan forklares ved et uundgåeligt randomiseret tab af albumin gennem denaturering hvor den endelige eliminering af det denaturerede albumin sker ved uspecifik proteolys enten direkte i extracellulærrummet eller på cytoplasmatiske lysosomer efter forudgående endocytose. Tillige sker der som vist gennem andres arbejde et tab ved udsivning fra organismen specielt gennem gastrointestinalepithelet.

## Acknowledgements

My sincere thanks are due to:

Tove Nerager my secretary for her skilful and devoted work and for loyal support through many years

Anna Margrethe Poulsen Knud Jørgensen Thes Gørtner Jørgen Jørgensen Svend Aage Kunz and Knud Pihl for skilful and co-operative technical assistance

Aase Evidt Henning Mouridsen and Jørgen Bojsen my co-authors for inspiring and pleasant collaboration

Poul Schack my good friend and colleague with whom I started out on my scientific career for his guidance and criticism which has helped towards a clear and precise final presentation of my work

Mogens Faber my boss for his enthusiasm and open mind and for giving me the opportunity to test my ideas in his laboratory

Francis Reithel and John Schellman University of Oregon Eugene Oregon USA for giving me opportunity to work in their laboratories and for valuable suggestions and discussions

Martin Ottesen The Carlsberg Laboratory for introducing me to the inspiring work of the late Kaj Ulrik Linderstrøm-Lang

The staff at the Pinsen Laboratory for encouragement and inspiration

Family and friends who with their never failing zest for the good life have delayed the publication of this work by several years

The work was supported by scholarships from the University of Copenhagen Fonden til Lægevidenskabens Fremme and The Danish Medical Council

# REFERENCES

- 1 Allison A C and P Davies Mechanisms of endocytosis and exocytosis In Transport at the Cellular Level Symp Soc Exp Biol Vol 28 pp 419-446 Eds M A Sleight and D H Jennings Cambridge University Press 1974
- 2 Andersen E B and N Rossing Metabolism of albumin and  $\gamma$ -globulin during albumin infusions and during plasmapheresis Scand J clin Lab Invest 1967 20 183-184
- 3 Andersson L O The heterogeneity of bovine serum albumin Biochim Biophys Acta 1966 117 115-133
- 4 Anfinsen C B Principles that govern the folding of protein chains Science 1973 181 223-230
- 5 Aoki K and J P Foster Electrophoretic behavior of bovine plasma albumin at low pH J Am Chem Soc 1957 79 5385-5396
- 6 Aoki K and J P Foster Limited hydrolysis of bovine plasma albumin at neutral and alkaline pH catalyzed by associated proteinases Biochemistry 1975 14 3566-3572
- 7 Behrens P O A M. Spiekerman and J R Brown Structure of human serum albumin Fed Proc (Fed Amer Soc Exp Biol ) 1975 34 591
- 8 Benacerraf B G Bionzi B N Halpern G Stiffel and D Mouton Phagocytosis of heat-denatured human serum albumin labelled with  $^{131}\text{I}$  and its use as a means of investigating liver blood flow Brit J Exp Path 1957 38 35-48
- 9 Bennett N S The concepts of membrane flow and membrane vesiculation as mechanism for active transport and ion pumping J Biophys Biochem Cytol 1956 2 Suppl 99-103
- 10 Berson S A and R S Yalow The distribution of I- $^{131}$  labeled human serum albumin introduced into ascitic fluid: Analysis of the kinetics of a three compartment catenary transfer system in man and speculations on possible sites of degradation J Clin Invest 1954 33 377-387
- 11 Blanchard J F N Tower D L Sorby and L D Tuck Investigation of drug-albumin interactions using spin-labeled bovine serum albumin Mol & Pharmacol 1975 11 138-143
- 12 Bloomfield V The structure of bovine serum albumin at low pH Biochemistry 1966 5 684-689

- 13 Bocsi V L Maati A Pacini and A Viti Catabolism of native and denatured serum 131-I-proteins by polymorph-nuclear leucocytes. *Exptl Cell Res* 1968 52 129-139
- 14 Bourdeau J E F A Carone and C E Ganote Serum albumin uptake in isolated perfused renal tubules. Quantitative and electronmicroscope radioautographic studies in three anatomical segments of the rabbit nephron. *J Cell Biol* 1972 54 382-398
- 15 Boyer P D F G Lam G A Ballou J M Lauck and R G Rice The combination of fatty acids and related compounds with serum albumin I Stabilisation against heat denaturation. *J Biol Chem* 1946 162 181-198
- 16 Bragdon J H and R S Gordon Jr Tissue distribution of C<sup>14</sup> after the intravenous injection of labeled chylomicrons and unesterified fatty acids in the rat. *J Clin Invest* 1958 37 574-578
- 17 Brambell P W R W A Hemmings and I G Morris A theoretical model of  $\gamma$ -globulin catabolism. *Nature (Lond)* 1964 203 1352-1355
- 18 Brambell P W R The transmission of immunity from mother to young and the catabolism of immunoglobulins. *Lancet* 1966 2 1087-1093
- 19 Brassinne A Gastric clearance of serum albumin in normal man and in certain gastroduodenal disorders. *Gut* 1974 15 194-199
- 20 Brown J R Structure of serum albumin: Disulfide bridges. *Fed Proc (Fed Amer Soc Exp Biol)* 1974 33 1389
- 21 Brown J R Structure of bovine serum albumin. *Fed Proc (Fed Amer Soc Exp Biol)* 1975 34 591
- 22 Brown J R Structure and evolution of serum albumin. In *Proceedings of FEBS (Federation of European Biochemical Societies) 11th Meeting Copenhagen 1977* Vol 50 pp 1-10. Eds T Peters and I Sjöholm. Pergamon Press Oxford 1978
- 23 Buys C H C M M G L Kliferink J M W Bouma M Gruber and P Nieuwenhuis Proteolysis and formaldehyde-treated albumin in Kupffer cells and its inhibition by suramin. *J Reticuloendothel Soc* 1973 14 209-223

- 24 Chapman-Andresen O Studies on pinocytosis in amoebae  
O H Trav Carlsberg Ser Chim 1962/63 33 73-264
- 25 Chapman-Andresen O Endocytosis in freshwater amoebae Phys  
Rev 1977 57 371-389
- 26 Chen R F Removal of fatty acids from serum albumin by char-  
coal treatment J Biol Chem 1967 242 173-181
- 27 Chiu W L and J H Haino Thermodynamic aspect of the inter-  
action of dichlorotetrafluoroethane with bovine albumin  
Pharmacology 1975 13 128-136
- 28 Coffey J W and C de Duve Digestive activity of lysosomes  
I The digestion of proteins by extracts of rat liver  
lysosomes J Biol Chem 1968 243 3255-3263
- 29 Cushman B W Structure-function relationships in the adipose  
cell II Pinocytosis and factors influencing its  
activity in the isolated adipose cell J Cell Biol  
1970 46 342-353
- 30 Davies M The effect of triton WR-1339 on the subcellular  
distribution of tryptan blue and 125-I-labelled  
albumin in rat liver Biochem J 1973 136 57-65
- 31 Eastman J W S J Rehfeld and H Loken Ultrafiltrable calcium  
and the conformation of albumin Clin Chim Acta 1975  
58 233-237
- 32 Edwards P B R M Rombauer and B J Campbell Thiol-disulfide  
interchange reactions between serum albumin and disul-  
fides Biochim Biophys Acta 1969 194 234-245
- 33 Ehrenreich B A and E Cohn The uptake and digestion of  
iodinated human serum albumin by macrophages in vitro  
J Exptl Med 1967 126 941-958
- 34 Eisenberg D and W Kauzmann The structure and properties of  
water Clarendon Press Oxford 1969 p 180
- 35 Fantl P Thiol distribution and mercaptalbumin reactivity in  
the blood plasma of different vertebrates Aust J Exp  
Biol Med Sci 1970 48 631-644
- 36 Flick A L and J L Steinf ld The effect of fever and cor-  
tiotropin on the in vivo degradation of albumin in  
man as measured with iodinated human serum albumin  
Am J Med Sci 1958 236 65-80

- 37 Foster J F M Sogami H A Petersen and W J Leonard Jr  
The microheterogeneity of plasma albumins I Critical  
evidence for and description of the microheterogeneity  
model J Biol Chem 1965 240 2495-2502
- 38 Franglen G and G R E Swaniker Preparation and analysis of  
peptide fragments produced by pepsin hydrolysis of  
human plasma albumin and their relationship to its  
structure Biochem J 1968 109 107-120
- 39 Freeman T The biological behaviour of normal and denatured  
human plasma albumin Clin Chim Acta 1959 4 788-792
- 40 Freeman T Analbuminaemia - a study of albumin and transferrin  
metabolism In Physiology and Pathophysiology of Plasma  
Protein Metabolism pp 75-85 Eds G Birke R Norberg  
and L O Plantin Pergamon Press Oxford 1969
- 41 Fuller-Noel J K and M J Hunter Bovine mercaptalbumin and  
non-mercaptalbumin monomers Interconversions and struc-  
tural differences J Biol Chem 1972 247 7391-7406
- 42 Gitlin J D and D Gitlin Cell receptors and the selective  
transfer of proteins from mother to young across tissue  
barriers Fed Res 1973 7 290
- 43 Gitlin D and J D Gitlin Genetic alterations in the plasma  
proteins of man In The Plasma Proteins 2 ed Vol 2  
pp 324-374 Ed F V Putnam Academic Press New York  
1975
- 44 Glenert J S Jarnum and S Riiser Experimental plasma pro-  
tein loss into the digestive tract in dogs Acta Chir  
Scand 1961 121 242-252
- 45 Glenert J S Jarnum and S Riiser The albumin transfer  
from blood to gastrointestinal tract in dogs Acta  
Chir Scand 1962 124 63-74
- 46 Goodman DeWitt H The distribution of fatty acids n-heptane  
and aqueous phosphate buffer J Am Chem Soc 1958 80  
3887-3892
- 47 Gordon A H and F Jacques Distribution of injected <sup>131</sup>I or  
<sup>125</sup>I labelled homologous plasma proteins among subcellu-  
lar particles of rat liver Proc Conf On Problems  
connected with the Preparation and Use of Labelled  
Proteins in Tracer Studies Pisa 1966 EURATOM  
Brussels 1966 (EUR 2950 d f e)

- 48 Gorini L and L Audrian Action de quelques métaux bivalents sur la sensibilité de la sérumalbumine à l'action de la trypsine *Biophys Biochim Acta* 1952 9 180-192
- 49 Harmsen B J M B H de Bruin L H M Janssen J P Rodrigues de Miranda and G A J Van Os pK change of imidazole groups in bovine serum albumin due to the conformational change at neutral pH *Biochemistry* 1971 10 3217-3221
- 50 Hawkins D R H Pinchard I P Crawford and H U Farr Structural changes in human serum albumin induced by ingestion of acetylsalicylic acid *J Clin Invest* 1969 48 535-542
- 51 Hoffenberg R A H Gordon E G Black and L N Louis Plasma protein catabolism by the perfused rat liver The effect of alteration of albumin concentration and dietary protein depletion *Biochem J* 1970 118 401-404
- 52 Holter H and H Holtzer Pinocytotic uptake of fluorescein-labelled proteins by various tissue cells *Exptl Cell Res* 1959 18 421-423
- 53 Hughes Jr W L Protein mercaptides In *Cold Spring Harbor Symposium on Quantitative Biology Vol 14: Amino Acids and Proteins* New York 1950
- 54 Hull H H E Chang and L J Kaplan On the location of the sulfhydryl group in bovine plasma albumin *Biochim Biophys Acta* 1975 400 132-136
- 55 Evidt Aa and B O Nielsen Hydrogen exchange in proteins *Adv Protein Chem* 1966 21 287-366
- 56 Ingram V M Gene mutations in human hemoglobin: The chemical difference between normal and sickle cell hemoglobin *Nature (Lond)* 1957 180 326-328
- 57 Jacobsen J Binding of bilirubin to human serum albumin Determination of the dissociation constants *FEBS Letters* 1969 5 112-114
- 58 Jacques P J Endocytosis In *Lysosomes in biology and pathology Vol 2 (Chapt 13)* pp 395-420 Eds J F Dingle and H B Fell North-Holland Publ Comp Amsterdam 1969
- 59 Jacques P J The endocytic uptake of macromolecules In *Pathologic Aspects of the Cell Membrane Chapt VII* Academic Press 1974
- 60 Jaffe E A R L Nachman C G Becker and C R Mini k Culture of human endothelial cells derived from Umbilical Veins *J Clin Invest* 1973 52 2745-2756



- 61 Janatová J On the heterogeneity of serum albumin A critical review J Med 1974 5 149-216
- 62 Janatová J J K Fuller and M J Hunter The heterogeneity of bovine albumin with respect to sulfhydryl and disulfide content J Biol Chem 1968 243 3612-3622
- 63 Jencks W P Catalysis in Chemistry and Enzymology" (Chapt 5) pp 296-298 Mc Graw-Hill New York 1969
- 64 Jencks W P Binding energy specificity and enzymic catalysis: The Circe effect Adv Enzymol 1975 43 219-410
- 65 Jocelyn P C The effect of glutathione on protein sulfhydryl groups in rat-liver homogenates Biochem J 1962 85 480-485
- 66 Jocelyn P C Biochemistry of the SH group Academic Press London 1972
- 67 Jones E A and T A Waldmann The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat J Clin Invest 1972 51 2916-2927
- 68 Katz J S Rosenfeld and A L Sellers Sites of plasma albumin catabolism in the rat Am J Physiol 1961 200 1301-1306
- 69 Keller H A Morell G Woseda and G Riva Analbuminämie Pathophysiologische Untersuchungen an einem Fall Schweiz Med Wochenschr 1972 102 71-78
- 70 Kerr R M J J du Bois and P R Holte Use of 125-I and 51-Cr-labeled albumin for the measurement of gastrointestinal and total albumin catabolism J Clin Invest 1967 46 2064-2082
- 71 King T P On the sulfhydryl group of human plasma albumin J Biol Chem 1961 236 P05
- 72 King T P and E M Spencer Amino acid sequences of the amino and the carboxyl terminal cyanogen bromide peptides of bovine plasma albumin Arch Biochem Biophys 1972 153 627-640
- 73 Kirsch R E L O C Frith and S J Saunders Albumin catabolism in vitro by cultured peritoneal and pulmonary mononuclear phagocytes Biochim Biophys Acta 1972 279 87-91
- 74 Klapper Jr J A and J R Cann Inhibition of peptic digestion of serum albumin by fatty acids Arch Biochem Biophys 1964 108 531-534

- 75 Kolthoff I M and B R Willeford Jr The interaction of copper (II) with bovine serum albumin J Am Chem Soc 1958 80 5673-5678
- 76 Kondo M Effect of fatty acids on the proteolysis of proteins III Hydrolysis of ovalbumin and serum albumin by several proteases in the presence of fatty acid J Biochem 1962 52 279-283
- 77 Laemmli U K Cleavage of structural proteins during the assembly of the head of bacteriophage  $\phi$  Nature (Lond) 1970 227 680-685
- 78 Laidler K J and P S Bunting The denaturation of proteins In The Chemical Kinetics of Enzyme Action 2 ed Chapt 13 pp 413-460 Clarendon Press Oxford 1973
- 79 Lakowicz J R and G Weber Quenching of protein fluorescence by oxygen Detection of structural fluctuations in proteins on the nanosecond time scale Biochemistry 1973 12 4171-4179
- 80 Lassen N A E H Parving and E Roseine Filtration as the main mechanism of overall transcapillary protein escape from the plasma Microvasc Res 1974 1 1-17
- 81 Laurell C B and E Thulin Thio-disulfide exchange in the binding of Bence Jones proteins to  $\gamma$ -globulin In the albumin and albumin J Exptl Med. 1973 137 453-465
- 82 Laurell C B and J O Jeppsson Protease inhibitors in plasma In The Plasma Proteins Vol 2 Pt 23-264 Ed F W Putnam Academic Press New York. 1974
- 83 Leibowitz M J and R L Soffer Enzymatic modification of proteins IV Site of acylation of bovine serum albumin in the leucine phenylalanine-transfer reaction J Biol Chem 1971 246 4431-4438
- 84 Leonard Jr W J K K Vijai and J P Foster A structural transformation in bovine and human plasma albumins in alkaline solution as revealed by rotary dispersion studies J Biol Chem 1963 238 1904-1914
- 85 Levy M and R B Warner Denaturation of bovine plasma albumin J Phys Chem 1954 58 106-109
- 86 Linderstrom-Lang K R D Hotchkiss and S Jansen Perturbations in globular proteins Nature (Lond) 1958 142 47

- 87 Linderstrom-Lang K U and J A Schellman Protein structure and enzyme activity In *The Enzymes* 2 ed pp 445-510 Eds P D Boyer H Lardy and K Myrback Academic Press New York 1959
- 88 Mackay M E and M H Martin The stabilization of purified human albumin to heat *Biochem J* 1957 65 284-288
- 89 McFarlane A S Metabolism of plasma proteins In *Mammalian Protein Metabolism* Vol 1 pp 297-341 Eds H N Munro and J A Allison Academic Press New York 1964
- 90 McFarlane A S and A Koj Short-term measurement of catabolic rates using iodine-labeled plasma proteins *J Clin Invest* 1970 49 1903-1911
- 91 McMenamy R H and J L Oncley The specific binding of L-tryptophan to serum albumin *J Biol Chem* 1958 233 1436-1447
- 92 Mego J L and J D McQueen The uptake of labeled proteins by particulate fractions of tumor and normal tissue after injection into mice *Cancer Res* 1965 25 865-869
- 93 Mego J L F Bertini and J D McQueen The use of formaldehyde-treated <sup>131</sup>I-albumin in the study of digestive vacuoles and some properties of these particles from mouse liver *J Cell Biol* 1967 32 699-707
- 94 Mehl T D and D Lagunoff Uptake of aggregated albumin by rat macrophages in vitro: Affinities of cells of monomeric and aggregated bovine serum albumin *J Reticuloendothel Soc* 1975 18 125-135
- 95 Moore A T K E Williams and J B Lloyd The effect of chemical treatment of <sup>125</sup>I-iodinated bovine serum albumin on its rate of pinocytosis by 17 5-day rat yolk sac *Biochem Soc Trans* 1974 2 648-650
- 96 Moore A T K E Williams and J B Lloyd The effect of chemical treatments of albumin and orosomucoid on rate of clearance from the rat bloodstream and rate of pinocytic capture by rat yolk sac cultured in vitro *Biochem J* 1977 164 607-616
- 97 Mouridsen H T Turnover of human serum albumin before and after operations *Clin Sci* 1967 33 345-354
- 98 Mouridsen H T and K Vallevik Metabolic and gel-electrophoretic properties of albumin isolated from wound tissue *Scand J Clin Lab Invest* 1968 22 322-327

- 99 Nikkel H J and J P Foster A  
lyzed structural alteration  
Biochemistry 1971 10 4479-4485
- 100 Norrman S J The kinetic of phar-  
clearance of denatured bovi-  
tive inhibition by denature-  
endothel Soc 1973 14 587-5
- 101 Palade G E The endoplasmic ret-  
Cytol 1956 2nd Suppl 85-90
- 102 Palmer D L D Rifkind and H P-  
human serum albumin in the s-  
system function: The distrib-  
-bound radionuclide J Lab Clin  
103 Pauling L H A Itano S J Singer  
cell anemia a molecular disea-  
543-548
- 104 Pedersen K O Exclusion chromatography  
phys 1962 Suppl. 1 157-168
- 105 Peterson E A and H A Sober Column  
teins: substituted celluloses  
Vol 5 pp 3-27 Eds S P Col-  
Academic Press New York 1962
- 106 Petitpierre-Gabathuler M -P and H J-  
uptake of soluble and aggregated  
between pinocytosis and phagocyt-  
19 141-156
- 107 Peters Jr T Serum albumin Adv Clin  
108 Peters Jr T Serum albumin In The V.  
Vol 1 pp 153-181 Ed F W Putnam  
New York 1973
- 109 Pfaff B M Schwenk R Burr and H Re-  
aspects of the interaction of bro-  
high affinity binding sites of bo-  
Mol Pharmacol 1975 11 144-152
- 110 Pickart L and M M Thaler Alt ration:  
tial of human albumin during stora-  
Res Commun 1977 74 961-968
- 111 Romsing N and H Jensen Metabolic sta-  
albumin preparations Clin Sci 19

- 112 Rossing N The normal metabolism of  $^{131}\text{I}$ -labelled albumin in man Clin Sci 1967 33 593-602
- 113 Rossing N "Human Albumin Metabolism Determined with Radio-iodinated Albumin Thesis Munkegaard Copenhagen 1971
- 114 Rotschild M A M Oratz and S S Schreiber Serum albumin Am J Dig Dis (New Ser ) 1969 14 711-744
- 115 Rotschild M A M Oratz and S S Schreiber Progress in gastroenterology Albumin metabolism Gastroenterology 1973 64 324-337
- 116 Rudolph R B Holler and R Jaenicke Fluorescence and stopped-flow studies on the N-F transition of serum-albumin Biophys Chem 1975 3 226-233
- 117 Byser E J O Aub and J B Caulfield Studies on protein uptake by isolated tumor cells II Quantitative data on the adsorption and uptake of  $^{131}\text{I}$ -serum albumin by Ehrlich ascites tumor cells J Cell Biol 1962 15 437-449
- 118 Saviotti M L and W C Galley Room temperature phosphorescence and the dynamic aspects of protein structure Proc Nat Acad Sci USA 1974 71 4154-4158
- 119 Schultze H E and J F Heremans Molecular Biology of Human Proteins Vol 1 Elsevier Publ Comp Amsterdam 1966
- 120 Simonescu R M Simonescu and G E Palade Permeability of muscle capillaries to small heme-peptides Evidence for the existence of patent transendothelial channels J Cell Biol 1975 64 586-607
- 121 Sogami M and J F Foster Microheterogeneity as the explanation for resolution of M and F forms of plasma albumin in electrophoresis and other experiments J Biol Chem 1963 238 PC2245-PC2247
- 122 Sogami M and J F Foster Isomerization reactions of charcoal-defatted bovine plasma albumin The N-F transition and acid expansion Biochemistry 1968 7 2172-2182
- 123 Sogami M H A Petersen and J F Foster The microheterogeneity of plasma albumins V Permutations in disulfide pairings as a probable source of microheterogeneity in bovine albumin Biochemistry 1969 8 49-58
- 124 Spahr F F and J T Edmull Amino acid composition of human and bovine serum mercaptalbumins J Biol Chem 1964 239 850-854

- 125 Spenner E M and T P King Isoelectric heterogeneity of bovine plasma albumin J Biol Chem 1971 246 201-208
- 126 Steinhardt J and J A Reynolds Multiple Equilibria in Proteins Academic Press New York 1969
- 127 Steinhardt J J B Leidy and J P Mooney Effects of n-alkyl ligands on the difference spectra of bovine and human serum albumin Biochemistry 1972 11 1809-1817
- 128 Steinman R M J M Silver and Z A Cohn Pinocytosis in fibroblasts J Cell Biol 1974 63 949-969
- 129 Sterling K The turnover rate of serum albumin in man as measured by I-131-tagged albumin J Clin Invest 1951 30 1228-1237
- 130 Stroupe B D and J F Foster Further studies of the sulfhydryl-catalyzed isomerization of bovine mercaptalbumin Biochemistry 1973 12 3824-3830
- 131 Svensson H Separation of proteins by isoelectric focusing in stable pH gradients Protides Biol Fluids 1967 15 515-522
- 132 Tanford C Protein denaturation Adv Protein Chem 1968 23 121-282
- 133 Tanford C Protein denaturation Theoretical models for the mechanism of denaturation Adv Protein Chem 1970 24 1-95
- 134 Trundle D and L V Cunningham Iodine oxidation of the sulfhydryl groups of creatine kinase Biochemistry 1969 8 1919-1925
- 135 Valdemann T A R D Wochner and W Strober The role of the gastrointestinal tract in plasma protein metabolism Am J Med 1969 46 275-285
- 136 Walker J E Lysine residue 199 of human serum albumin is modified by acetylsalicylic acid FEBS Lett 1976 66 173-175
- 137 Vallevik K Thermodynamic of the denaturation of human serum albumin in relation to intra-vital breakdown Protides Biol Fluids 1972 19 429-436
- 138 Varner R G and M Levy Denaturation of bovine plasma albumin II Isolation of intermediates and mechanism of the reaction at pH 7 J Am Chem Soc 1958 80 5735-5744
- 139 Watson J H Molecular Biology of the Gene W A Benjamin Inc pp 102-140 New York 1970

- 140 Williams K E J B Lloyd M Davies and F Beck Digestion of an exogenous protein by rat yolk-sac cultured in vitro Biochem J 1971 125 303-308
- 141 Williams K E E M Kidston F Beck and J B Lloyd Quantitative studies of pinocytosis II Kinetics of protein uptake and digestion by rat yolk-sac cultured in vitro J Cell biol 1975 64 123-134
- 142 Yutani K K Ogasahara Y Sugino and A Matsushiro Effect of a single amino acid substitution on stability of conformation of a protein Nature (Lond ) 1977 267 274-275
- 143 Zurawski V P and J R Foster The neutral transition and the environment of the sulfhydryl side chain of bovine plasma albumin Biochemistry 1974 13 3465-3471

f

t

—

2

—





**TRANSCAPILLARY SOLUTE EXCHANGE IN SKELETAL MUSCLE  
AFTER INJURY AND DURING SHOCK**

By  
**BERTIL DAHLBERG**



From the Department of Surgery I, Sahlgren's Hospital, University of Göteborg, Göteborg,  
Sweden

**TRANSCAPILLARY SOLUTE EXCHANGE IN SKELETAL MUSCLE  
AFTER INJURY AND DURING SHOCK**

By  
**BERTIL DAHLBERG**

DAHLBERG J.B. *Transcapillary solute exchange in skeletal muscle after injury and during shock.* Acta physiol. scand. 1979 Suppl. 472. 1-82.

The specific object of the investigation was to study blood flow and blood tissue exchange of small molecules in canine skeletal muscle during hemorrhagic shock, after muscle injury and in normovolemic hemodilution. The effects of regional arterial hypotension and of increased venous pressure were also studied in order to acquire additional information about skeletal blood circulation as it functions in low flow states. The non-isolated hind leg musculature was used throughout the studies except in a few experiments, where the hind leg was isolated in order to estimate the capacity of collateral veins. The principal technique employed to study the blood tissue exchange was the single-injection multiple-indicator dilution method. A local clearance method was used simultaneously in some of the experiments on hemorrhagic shock and normovolemic hemodilution. Cr-EDTA was the principal diffusible lipid insoluble indicator for both methods. I-antipyrine was used in the majority of the experiments with the indicator dilution method as lipid soluble test molecule. Fe-dextran was the intravascular marker. The capillary blood flow rate in the local clearance method was taken as Xenon clearance. Isotope radioactivities in blood samples were measured in a well-type scintillation counter in muscle deposits by external detection. Regional arterial hypotension (50 mm Hg) was produced by partial occlusion of the aorta. Hemorrhagic shock was produced by repeated arterial bleedings to maintain arterial pressure around 50 mm Hg. Muscle injury was produced by a series of hammer strokes on the thigh. Normovolemic hemodilution was achieved by replacing extracorporeal volumes of whole blood with equal amounts of dextran 70 (hematocrit at level 1: 25-30 %, at level 2: 10-12 %). Venous pressure was elevated (10 mm Hg) by graded occlusion of the iliac vein. Total blood flow was measured from the timed venous outflow. Values for extraction (E) of diffusible isotopes were used for lipid insoluble test solutes, to calculate the permeability-surface area product (PS) if blood flow (total blood flow or capillary blood flow) is denoted by Q and the natural logarithm by  $\ln$ ,  $PS = -Q \ln(1-E)$ . The evaluation of the results was promoted by a computerized statistical analysis of the data, including the assembled data from the control, i.e. the basal states. The extraction of I-antipyrine, 0.79 was independent of changes in blood flow rate, induced in the different activated states. PS found (for Cr-EDTA) to change with flow decreased as a result of severe hemorrhage; this was the case for all lipid insoluble indicators ( $^{125}I$ ,  $^{14}C$ ,  $^{51}Cr$ -EDTA) used with the indicator dilution method. PS(Cr-EDTA) was reduced by 50 % after two hours of hemorrhagic hypotension. The magnitude of this decrease in PS was the same when estimated by the local clearance method. Following injury total blood flow either increased or decreased. PS(Cr-EDTA) decreased. The decrease in plasma flow seen at both levels of normovolemic hemodilution (dextran 70) and registered by both the indicator dilution and the local clearance method was accompanied by an increase in PS(Cr-EDTA) when the measurements were made by the indicator dilution method, no change in PS(Cr-EDTA) was registered when measured simultaneously by the local clearance method. Following graded occlusion of the iliac vein, a part of the venous return was diverted into collateral blood vessels leading from the hindleg; the elevation of venous pressure did not appear to influence the PS value for Cr-EDTA. It was concluded that, when the blood flow in the muscle decreases as a consequence of partial occlusion of the aorta, severe hemorrhage or injury, the PS values for lipid insoluble solutes also decrease. It was also concluded that an increase in flow, as seen during normovolemic hemodilution with dextran 70 may be accompanied by an increase in PS but also, as seen after injury by a PS reduction. Finally it was concluded that elevation of venous pressure does not affect the passage across the capillary wall of small solutes like Cr-EDTA.

Copyright © 1979  
by Acta Physiologica Scandinavica.  
All right reserved.  
Printed in Sweden.  
ISSN 0302-2994

Acta Physiologica Scandinavica  
c/o Almqvist & Wiksell Tryckeri AB  
Reprintum 8  
751 01 Uppsala, Sweden

## CONTENTS

3	INTRODUCTION
3	AIM OF THE PRESENT STUDY
4	Chapter 1
4	BACKGROUND
4	A. Regulation of peripheral circulation and of solute and water flows across the capillary wall
4	B. Methods for measuring transcapillary exchange
5	1. The single injection indicator dilution method
6	2. The local tissue clearance method
7	C. Some current concepts on blood tissue exchange in states of disturbed circulation
7	1. Regional arterial hypotension
7	2. Haemorrhage
8	3. Injury
9	4. Haemodilution
9	5. Increased venous pressure
10	6. The effect of surgical preparation
11	Chapter 2
11	MATERIAL AND METHODS
11	Experimental procedures
12	Isotopes
12	Measurement of radioactivity
12	Experimental procedures for the indicator techniques
12	1. The single injection multiple indicator dilution method
13	2. The local clearance method
15	Presentation of the relations between extraction (E), blood flow (Q) and permeability-surface area product (PS)
16	Statistical methods
19	Chapter 3
19	ANALYSIS OF VENO-CONCENTRATION AND EXTRACTION CURVES (INDICATOR DILUTION METHOD)
19	Results
19	Comments
22	Summary
23	Chapter 4
23	DESCRIPTION OF THE CONTROL STATE (INDICATOR DILUTION METHOD)
23	Methods
23	Results
24	Comment. The use of the control state for the evaluation of the activated state
27	Chapter 5
27	BLOOD TISSUE EXCHANGE DURING REGIONAL ARTERIAL HYPOTENSION
27	Experimental procedures
27	Results
30	Comments on results
30	Summary

31	Chapter 6
31	BLOOD TISSUE EXCHANGE DURING HEMORRHAGIC HYPOTENSION
31	Experimental procedure
32	Results
36	Comments on results
36	Summary
37	Chapter 7
37	BLOOD FLOW AND BLOOD TISSUE EXCHANGE IN TRAUMATIZED SKELETAL MUSCLE
37	Experimental procedure
37	Results
42	Comments on results
42	Summary
43	Chapter 8
43	BLOOD FLOW AND BLOOD TISSUE EXCHANGE DURING NORMO-VOLEMIC REMODULATION
43	Experimental procedure
43	Results
48	Comments on results
48	Summary
49	Chapter 9
49	INFLUENCE OF INCREASED VENOUS PRESSURE ON TOTAL BLOOD FLOW AND BLOOD TISSUE EXCHANGE IN THE DOG HIND LEG
49	Experimental procedure
51	Results
54	Comments on results
54	Summary
55	Chapter 10
55	GENERAL DISCUSSION
55	A. Methodological considerations
55	1. Blood flow Q
56	2. Extractions, E
56	a) I-nitroprusside a lipid-soluble molecule
56	b) Cr-EDTA, lipid-insoluble (water-soluble) molecule
57	3. FI, FI/Q quotient of Cr-EDTA or isolate and back-diffusion
58	4. Blood to tissue vs tissue to blood transport
58	B. The control state
59	C. The activated state
59	1. The activated state with a decreased blood flow
59	a) Regional arterial hypotension
60	b) Hemorrhagic hypotension
62	2. The activated state with a variable blood flow
62	Ischemy
64	3. The activated state with an increased blood flow
64	Acute normovolemic hemodilution
64	4. Increased venous pressure
67	SUMMARY AND CONCLUSIONS
69	ACKNOWLEDGMENTS
71	REFERENCES

## INTRODUCTION

The vascular bed in skeletal muscle is of very great importance in the regulation of the general circulation and for the satisfaction of increased metabolic demands occurring, e.g. during muscular work or in disease.

Much data have been accumulated over the latest century about the basic morphological and physiological properties of this vascularity. The methods which have been employed to study the capillary blood circulation in skeletal muscle have rarely been free from difficulties. Some difficulties have arisen as a result of the large variability of peripheral blood flow and its relation to the variety of different properties of the capillary wall and its adjunctive tissue. They have been evident, in particular, when diffusion exchange across the capillary wall has been studied in whole organs.

The degree of variation which characterizes many events in a vascular bed may often be raised in *pathological* states. This could add to the variety of difficulties involved when the circulatory response to *injury* is to be studied and could help to explain why so much uncertainty remains about capillary permeability and blood-tissue exchange, e.g. in skeletal muscle after injury and during various kinds of shock.

## AIM OF THE PRESENT STUDY

The purpose of the present investigation was to study the blood flow response and the blood tissue exchange of small molecules in dog skeletal musculature during shock. The single-injection multiple-indicator dilution method was used to measure the transcapillary solute flow from *blood to tissue* in some conditions of surgical interest, where the skeletal blood circulation is greatly affected. The technique was employed for studies on hemorrhagic shock, tissue trauma and hemodilution. In the hemodilution experiments no deliberate attempt was made to produce a shock-like condition in the animal prior to the hemodilution.

To elucidate such aspects as hypotension in shock and venous stasis in trauma additional studies were performed on the effects of regional arterial hypotension and of regional ly increased venous pressure.

A local clearance method was used simultaneously in the series of experiments on hemorrhagic shock and on hemodilution to measure *tissue to blood* transport.

Throughout the study it was assumed that total blood flow from the hind leg musculature which was under investigation, was represented by the flow from the flac vein and, further, that this blood flow represented the total capillary blood flow through the musculature. The first assumption was tested by comparing arterial inflow and venous out flow in some experiments at normal and increased venous pressure.



## BACKGROUND

## A. Regulation of peripheral circulation and of solute and water flows across the capillary wall

The mechanisms by which the peripheral circulation is regulated are sensitive both to the influence of locally situated control systems within the peripheral tissue and blood vessel walls and to centrally generated nervous and humoral control. The interaction of these control systems is not only regulating the peripheral blood perfusion but also the exchange processes between blood and tissue (e.g. Renkin 1964, Mellander 1960, 1971). These processes are relatively more sensitive to influence of the local control mechanisms because of the particular sensitivity of the muscle tone of the precapillary sphincters to changes in local metabolism and blood pressure variations (Mellander and Johansson 1968, Mellander 1970). The nervous, mainly vasoconstrictor influence on the capillary exchange area on the other hand is less sustainable but of particular importance for fluid absorption by its decreasing effect on the capillary pressure (Cobbold et al 1963, Öberg 1964).

The diffusion flow of water soluble (lipid-insoluble) solutes, mainly confined to pore pathways depends on the capillary permeability( $P$ )-surface area( $S$ ) product( $PS$ ) and on concentration gradients across the capillary wall. The magnitude and importance of convection exchange of solutes across the capillary wall are much debated. It seems to increase with molecular size so that for large solute molecules it may even predominate over dissipative transport especially at abnormally high rates of net volume flow across the capillary wall (Renkin et al 1977a, b, Rippe et al 1979).

In contrast small lipid-soluble molecules, such as Xenon and enthalpyrine will meet practically no barrier for transport at the capillary wall.

The exchange of water is basically regulated by the capillary hydrostatic pressure and the plasma protein osmotic pressure (Starling 1896, Landis 1927). The capillary pressure depending on the post to precapillary resistance ratio tends to increase in vasodilatation (Pappenheimer and Soto-Rivera 1948). Water is crossing the capillary wall via pathways through the endothelial cell membrane and via intercellular pathways, both small and large (e.g. Curry et al 1976, Renkin and Curry 1978).

The presence of plasma proteins is also necessary for the maintenance of a normal capillary permeability (e.g. Danielli and Stock 1944, Levick and Michel 1970, Curry et al 1973, Mason et al 1973, 1977).

## B. Methods for measuring transcapillary exchange

In order to analyse the effects of capillary blood flow on diffusion transport of solutes

models of the capillary or the capillary bed have been devised (Krogh 1919; Renkin 1957; Renkin 1959; cf. Reviews and Models). The construction of such models necessitated several assumptions concerning the structure and function of the capillary bed. In the single capillary model, which was originally used to formulate a relationship between the product of capillary permeability and surface area (PS) on one hand and flow and solute fractional extraction (E) on the other:

$$PS = -Q \ln(1-E) \quad (\text{Petersen 1967})$$

It is assumed that the permeability along the capillary is uniform and that there is a progressive concentration fall of a solute moving along the capillary. Any diffusion gradients or permeability barriers outside the capillary are not accounted for (cf. Crook and Petersen 1970). When the formula is used for studies on an entire capillary bed rather than a single capillary one has to presuppose that there is uniformity between all the capillaries with respect to their individual exchange/blood flow ratio (or  $ps/q$  ratio). The value of PS and  $PS/Q$  calculated from extraction and flow for the whole vascular bed where extraction (E) will be a flow weighted mean value of extractions  $[1 - \exp(-ps/q)]$  in different parts of the capillary bed may hide complex patterns of distribution of  $ps/q$ .

### 1 The single-injection indicator-dilution method

This method originally devised by Chinard and colleagues (1955) is a modification of the technique of Stewart (1897; 1921a, b) and Hamilton (Kinman et al. 1932) for measuring blood flow. It has been extensively employed in various organs and tissues (see separate list of references). With few exceptions, the method has only been applied in studies on normal physiological states. It has been done in skeletal muscle during injury or disturbed circulation with few exceptions.

The technique is based on the measurement of the fractional extraction of a substance from the blood into the tissue during the first passage through the capillary bed. The extraction is derived from concentrations at the venous outflow of tracers as fractions of an indiffusible tracer injected simultaneously into the arterial blood. With the single capillary model, it is assumed that there is an exponential concentration fall of the diffusible indicator along the capillary (Krogh) and that this indicator is dispersed intravascularly to the same extent as the other indicator.

$$C_v/C_a = \exp(-PS/Q)$$

$C_v$  is the venous concentration of the diffusible indicator,  $C_a$  the arterial concentration of the indiffusible indicator,  $P$  is the capillary permeability,  $S$  is the capillary area available for indicator diffusion,  $Q$  is the blood flow through the organ, assumed to be equal to the total blood flow through the organ. The assumption that the indicators are uniformly dispersed intravascularly is a simplification.

of error since it is known that the indicator molecules of different molecular weights disperse differently (Taylor 1954).

The fractional extraction (E) of the diffusible indicator is  $(C_2 - C_1) / (C_2 - C_1)$ . The tissue concentration of the tested solute is supposed to be so low that no back-diffusion takes place during the early stage of solute passage. This is the case if  $C_1$  the tissue concentration of the diffusible indicator is zero during the early phase of the indicator passage through the organ (Crone 1963, Martín de Jullán and Yudilevich 1964, Yudilevich et al. 1968). PS may then be calculated from the equation  $PS = Q \ln(1-E)$  without correction for back-diffusion.

More extensive calculations, taking into account the whole period of exchange after injection, including back-diffusion, have been applied, e.g. by Goresky and Bassingthwaite and their colleagues (1963, 1970, 1970, 1974 cf. Methods and Theory). These methods (see e.g. Güller et al. 1975) are beyond the scope of the present study.

In order to avoid the difficulties concerning non-uniformity of extraction values among the capillaries within the vascular bed and flow-limitation of transcapillary exchange, the method is often employed at high rates of blood flow which, in practice, implies a PS/Q ratio less than 0.5 (Goresky et al. 1970, Bassingthwaite 1974, Güller et al. 1975, Rippe 1978). As mentioned earlier, the formula is based on the assumption that the fractional extraction is the same in all the capillaries of the capillary bed.

The transcapillary passage of the lipid soluble antipyrine is rapid in relation to blood flow. Its extraction is therefore expected to be constant at various flow levels, indicating a flow-limited clearance (Renkin 1952, 1953).

## 2. The local tissue clearance method

This modification of the tissue clearance method (Kety 1949) was devised to study the capillary permeability-surface area product (PS) by simultaneous clearances of an inert gas and a hydrophilic ion (Gosselin 1967, Lassen and Trap-Jensen 1968, Appelgren and Lewis 1968, Strandell and Shepherd 1968). It is assumed that no diffusion gradients in the interstitial space will develop at any time during the washout period and that there is uniformity of the capillary bed.

The method is based on the assumption that the fractional washout rate  $(dA/dt) \times (1/A)$  from the tissue interstitium is constant both for the hydrophilic small molecule (as Cr-EDTA) and the lipophilic gas (as Xenon) for a period following the simultaneous local injection into the tissue. When the local tissue clearance method is employed it is also necessary to assume that the interstitial volume constitutes a specified percentage of the tissue and that there is a uniformity in the capillary bed. Osmotic effects of the injectate are, as in the indicator dilution method, considered to be low enough to be disregarded.

Xenon, an inert gas, is expected to equilibrate with blood during its passage through the tissue giving a Xenon clearance equalling the blood flow Q.

Calculation of clearance and extraction is made according to the equations

$$dA/dt = -k \times A$$

$$C = \lambda \times 100 \times k \quad \text{ml/min/100 g tissue}$$

$$E = C/Q = 1 - \exp(-PS/Q)$$

where  $k$  is the fractional washout rate in  $\text{min}^{-1}$  and  $\lambda$  the tissue-blood partition coefficient.  $A$  is the (relative) counts per minute of the residual radioactive activity in the indicator depot.

The extraction of the hydrophilic molecule is the ratio of the clearance values of this substance to that of the gas, if the gas clearance value indicates blood flow ( $E = C/Q$ ). For some molecules diffusion gradients are likely to arise in the interstitium, although such effects depend on the molecular size of the hydrophilic substance and seem to be minimized for certain substances, e.g.  $^{51}\text{Cr}$ -EDTA (Mw = 341) (Lassen and Trap-Jensen 1970).

## C Some current concepts on blood tissue exchange in states of disturbed circulation

### 1 Regional arterial hypotension

Renkin (1959) found a systematic variation in measured permeability-surface area product (PS) with changes in blood flow and vascular resistance indicating that the capillary circulation is not uniform and that observed PS values principally refer to transcapillary exchange in well-perfused blood vessels.

Renkin's observations have been confirmed in several studies on blood tissue exchange (Trap-Jensen and Lassen 1970, Appelgren 1972, Lind, Chihholm and Rosell 1974, Paasik 1977, Rippe, Kamiya and Folkow 1978). This systematic relationship between observed PS values and rates of blood flow was seen even when the vascular bed is dilated. It therefore seems likely that stagnancy of capillary blood flow may have similar effects on blood tissue diffusion exchange as actual closing or corpuscular obstruction of parts of the capillary bed.

### 2. Hemorrhage

Acute hypovolemia elicits a great variety of reactions concerning the blood circulation. Some of the reactions, such as those affecting vascular adaptation and fluid transfers between tissue compartments, are comparatively well known, both in a qualitative and in a quantitative respect (see Reviews). Some of the changes seen in hemorrhagic shock may be caused solely by the reduction of blood flow whereas others apparently occur as a consequence of an altered distribution of capillary blood flow (Appelgren 1972). The redistribution of blood flow does not seem to be caused only by compensatory reflex mech-

animals, responding to decreased blood flow and perfusion pressure (Mellander and Lewis 1963 Chien 1967 1969 Mellander and Öberg 1967 Järhult et al. 1972, Järhult 1973 1975). It also appears to be a less adaptive response, due to the early development, in shock, of a large scale capillary imperviousness or stasis, mainly caused by obstructing blood corpuscles and other rheological factors.

Data indicating such changes have been obtained in studies on the capillary filtration coefficient, giving the impression that extensive areas of the capillary bed in muscle become obstructed after hemorrhage (Baeckström et al. 1971).

The transport of solutes from the interstitial fluid, expressed as capillary diffusion capacity (PS), to the blood has also been found to become greatly reduced during hemorrhagic shock (Appelgren 1972). At perfusion pressures above 60 mm Hg, the reduction was equivalent to a 50 % decrease of the permeability surface area product (PS) in comparison with control values at similar perfusion pressures. This finding was interpreted as being due to a reduction of the number of perfused capillaries. An additional reversible, reduction of PS was seen at variation of perfusion pressures within the 20 - 60 mm Hg range coinciding with a similar kind of response in PS of controls. It was proposed that such a PS reduction in shock at low perfusion levels can be explained by an increasing heterogeneity of the residual capillary blood flow

## Injury

Landis (1934) described the complex response that local injury initiates: vasodilatation, rise in capillary blood pressure first increased, then decreased blood flow increased endothelial permeability and finally stasis. In their review in *Handbook of Physiology* (1963) Landis and Pappenheimer knowing that diffusion rates had not been previously measured when effects of severe injury were studied, presumed one should expect an increased net diffusion of molecules across the capillary wall as long as blood flow continues (at an unchanged or even increased rate) but that the effectiveness of diffusion, in exchanges of substances would decline as flow decreases or ceases, since capillary exchange would then become flow limited. Landis (1927) found, when studying the increased permeability in injured capillaries, that increase of the capillary blood pressure was a more consistent response to injury than vasodilatation.

Regional contusion of the dog's skeletal musculature has often been seen to cause an immediate local vasodilatation (Liu 1968 Lewis and Lim 1970, Sandegård 1974). This hyperaemia is assumed to have certain characteristics in common with metabolic vasodilatation (exercise or functional hyperaemia and reactive hyperaemia following temporary arterial occlusion) in that the myogenic tone of the precapillary resistance vessels is presumed to diminish by the influence of locally accumulated metabolites. In reactive hyperaemia the myogenic tone is also influenced by perfusion pressure change since blood pressure reduction, like tissue hyperosmolality inhibits the myogenic pacemaker

activity in the walls of precapillary resistance vessels. Locally accumulated metabolites during muscle exercise or a decreased blood flow produce vasodilatation by similar mechanisms tissue hyperosmolality hypoxia and extracellular potassium are all known to reduce the tone in both precapillary resistance vessels and sphincters (e.g. Johnson 1974).

#### 4 Hemodilution

Reduction of the plasma volume and disturbance of the capillary blood flow are common features in various states of shock. Plasma substitutes, often primarily administered to restore the intravascular volume of blood plasma, may also influence the distribution of the capillary blood flow and, in consequence of that, the blood-tissue exchange of water and water soluble molecules.

Solutions of dextran 40 and dextran 70 have been shown, in particular to possess beneficial properties as plasma substitutes in shock (Gelin 1962a, b 1973 Lim et al. 1968 Dawidson et al. 1979b c). The blood tissue diffusion exchange of small hydrophilic molecules has been found to improve, in shock, after administration of dextran 40 (Appelgren 1972). Comparative studies are currently under progress to examine the properties of various kinds of infusates, including dextran 40 and dextran 70, with respect to their influence on transcapillary solute exchange and tissue  $O_2$  tension in skeletal muscle during shock (Dawidson et al. 1979a). The disturbance of capillary circulation, which is seen to follow arterial hypotension (cf. Järhult and Mellander 1974) appears to be markedly alleviated by dextran 40 (Appelgren 1972).

The mentioned dextran solutions seem to counteract the non-uniformity and obstruction of capillary blood flow which follow hypotension and shock, thus establishing a larger "effective" capillary surface area available for diffusion transport. The exact mechanisms, by which such an improvement is achieved are not, as yet, fully understood.

#### 5 Increased venous pressure

It could be argued that an extensive preparation procedure, apart from having possible side-effects on the distribution of capillary blood flow may also affect the blood flow through the major pelvic veins by exposing these vessels to an increased external pressure. The effect of an increased venous pressure on blood flow and transcapillary solute exchange would depend on

- a) the opening of alternative routes for the blood returning from the hind leg, e.g. collateral veins,
- b) possible changes of total capillary blood flow and capillary surface area and
- c) fluid movements from blood to tissue induced by an increased capillary pressure

It has only occasionally been proposed that solvent drag can account for any considerable part of the transport of small lipid insoluble molecules (Åberg 1973). Pappenheimer et al. (1951) calculated the relations between diffusion and filtration transfer for various

1. The first part of the document is a letter from the President of the United States to the Congress, dated January 1, 1861. It is a formal communication, and it is written in a very formal and dignified style. The President expresses his regret that he cannot continue to serve the country, and he expresses his confidence in the future of the country.

1. The first step is to identify the problem or question that needs to be answered.

[illegible]

## MATERIAL AND METHODS

The experiments were carried out on 78 adult mongrel dogs of both sexes (9.5 to 28 kg). Splenectomy was performed at the initial stage of the experiments on hemodilution. Eleven experiments were discarded due to untimely animal deaths or to technical errors. One dog was exclusively used to test the experimental set-up and the isotope injection and blood-sampling techniques. Three dogs were used entirely for comparative blood-flow measurements.

### Experimental procedures

The dogs were anaesthetized with sodium pentobarbital intravenously. Small supplement ary doses were given as required. The airway was secured by an endotracheal tube. In the series of experiments on trauma the breathing was further supported and controlled by a respirator. The animals were not heparinized. The vascular bed of the hind leg musculature was not vasodilated.

### Infusates

Saline was slowly infused intravenously throughout the experiments via a catheter to one jugular vein.

Six per cent dextran 70 (Macrodex® Mw about 70000 supplied by Pharmacia, Sweden) was used to achieve normovolemic hemodilution.

### Pressure and flow measurements

Arterial blood pressure was measured by a mercury manometer via a catheter from one carotid artery and also in the experiments on regional arterial hypotension, from the sacral artery.

Venous blood pressure was measured by a water manometer via a wide bore siliconized plastic T-tube placed in one iliac vein.

The venous return was diverted through the T tube for serial blood sampling (see indicator-dilution technique) and was timed in order to measure blood flow. The weight of the hind leg was registered at the end of each experiment for conversion of blood flow to ml/min/100 g.

Regional arterial blood flow was directly measured in a few experiments in order to compare arterial blood flow into the hind leg with venous outflow (for a detailed description see Chapter 9). An electromagnetic flow meter (Nycotron, Oslo, Norway) was used with the probes placed on the external iliac artery proximal to the deep femoral artery.



## Isotopes

### I For indicator-dilution method

#### *Diffusible*

Water soluble lipid insoluble extracellularly distributed

- a)  $^{51}\text{Cr}$ -EDTA. T  $1/2$  ( $^{51}\text{Cr}$ ) 27.8 d.  $\gamma$ -energy ( $^{51}\text{Cr}$ ) 0.320 MeV Mw 341
- b)  $^{125}\text{I}$ iodide T  $1/2$  60 d.  $\gamma$ -energy 0.035 MeV
- c)  $^{131}\text{I}$ iodide T  $1/2$  8.04 d.  $\gamma$ -energy 0.364 MeV

Lipid soluble intracellularly distributed

$^{125}\text{I}$  and pyrene.

Monthly prepared and dispatched, free iodide less than 5 %. Mw 312.2.

#### *Non-diffusible*

- a)  $^{59}\text{Fe}$ -siderophillin. T  $1/2$  ( $^{59}\text{Fe}$ ) 45 d.  $\gamma$ -energy ( $^{59}\text{Fe}$ ) 1.10 MeV

Prepared by mixing  $^{59}\text{FeCl}_3$  with dog's plasma.

- b)  $^{125}\text{I}$ -RIHSA Free iodide less than 5 %

### II. For local clearance method

$^{51}\text{Cr}$ -EDTA (see I).

$^{133}\text{Xe}$ on. T  $1/2$  5.3 d.  $\gamma$ -energy 0.081 MeV Dissolved in saline solution. 0.1 ml injected. Specific activity 2 – 10 Ci/cm<sup>3</sup> Xe at stp

## Measurement of radioactivity

The radioactivity of blood samples was measured in a well type scintillation counter with a  $\gamma$ -spectrometer. The three isotopes,  $^{125}\text{I}$ ,  $^{51}\text{Cr}$  and  $^{59}\text{Fe}$  were measured on three different channels. Corrections for background and for interferences between the various isotopes were made. The radioactivity from isotope depots in muscle tissue was measured by a single scintillation counter with a two-channel spectrometer with ratemeters and recorders.

Hematocrit was measured by microcapillary centrifugation and used in the calculation of tissue-blood partition coefficients of Xenon (Andersen and Ladefoged 1967) and of  $^{51}\text{Cr}$ -EDTA (Appelgren 1972b).

## Experimental procedure for the indicator techniques

### 1 The single injection multiple indicator dilution method

In the present study the indicator dilution method was used for mea

of the blood tissue exchange in a non-vasodilated vascular bed. Following laparotomy the vasculature to one hind leg of the dog was freed from above the bifurcation of the aorta to the inguinal ligament. The blood circulation of the paw was excluded by a ligature around the ankle. A catheter was introduced into the iliac artery via a small sidebranch. A mixture was prepared of dog plasma and appropriate amounts of isotopes (approx. 150 – 250  $\mu\text{Ci}$  for  $^{51}\text{Cr}$ -EDTA, 100 – 150  $\mu\text{Ci}$  for  $^{125}\text{I}$ -antipyrine 15 – 20  $\mu\text{Ci}$  for  $^{59}\text{FeCl}_3$ ). One ml of this mixture was used for each injection which was rapidly made into the artery with a syringe. The blood flow from the iliac vein was, via a wide-bore catheter collected in vials, placed in a fractional collector (Fig. 1). Collection time varied between 20 seconds and 2 minutes depending on flow rate. The amount of the various isotopes in the injectate was measured at the end of each experiment and used to normalize the time-concentration curves that were constructed for each isotope. Normalizing was achieved by calculating the concentration of various isotopes in the fractional blood samples, expressing the concentration as per cent of given amount in the injectate per gram blood (Fig. 2). Radioactive background activity was measured from early blood samples collected before the injectate started to reach the collector.  $^{59}\text{FeCl}_3$  was used in all experiments as a vascular marker. It was assumed that this isotope when binding irreversibly to siderophilin, functions as an indiffusible indicator (Yudilevich and Martin de Julián 1965).

The diffusible isotopes used were iodide ion ( $^{131}\text{I}$  or  $^{125}\text{I}$ ) or, most commonly  $^{51}\text{Cr}$ -EDTA together with  $^{125}\text{I}$ -antipyrine.  $^{125}\text{I}$ -RIHSA was used in two experiments for comparison with  $^{59}\text{Fe}$ -siderophilin.

Extractions of diffusible isotopes were calculated as  $E = (C_{\text{nondiff}} - C_{\text{diff}}) / C_{\text{nondiff}}$ , where  $C_{\text{nondiff}}$  stands for concentration of  $^{59}\text{Fe}$ -siderophilin and  $C_{\text{diff}}$  for either  $^{51}\text{Cr}$ -EDTA,  $^{125}\text{I}$  or  $^{131}\text{I}$  or  $^{125}\text{I}$  antipyrine, after standardization of isotope concentrations for injected amount of the isotopes in the arterial bolus (Fig. 3).

## 2. The local clearance method

$^{133}\text{Xe}$  and  $^{51}\text{Cr}$ -EDTA were mixed and 0.1 ml of the mixture slowly injected into the muscle. The injection needle remained in the muscle for 30 seconds following the injection. The radioactivities at the injection site were counted by a scintillation detector and recorded. After corrections for background activity ( $^{51}\text{Cr}$ -EDTA) and interference ( $^{133}\text{Xe}$ ) the radioactivities as counts per minute were plotted semilogarithmically against time.  $k$ -values were calculated from  $t_{1/2}$  of the monoexponential part of the curve. Clearances were calculated from  $k$ -values and the partition coefficients. Extraction of  $^{51}\text{Cr}$ -EDTA was estimated as the ratio between the clearances of this substance and  $^{133}\text{Xe}$  and subsequently used in the formula  $E = (C/Q) / (1 - \exp(-PS/Q))$ .

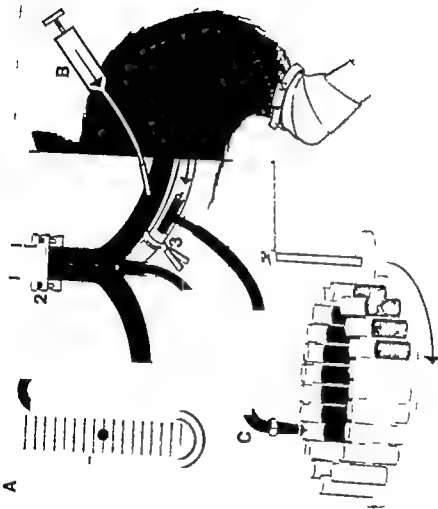


Fig. 1 Diagram of the experimental set-up for indicator dilution studies on a rat during regional arterial hypotension produced by constriction of the aorta. (1) return in the iliac vein (occluded at 3) is sampled in vitro placed on rotating

1272 arterial pressure  
 1273 arterial pressure  
 1274 arterial pressure  
 1275 arterial pressure  
 1276 arterial pressure  
 1277 arterial pressure  
 1278 arterial pressure  
 1279 arterial pressure  
 1280 arterial pressure  
 1281 arterial pressure  
 1282 arterial pressure  
 1283 arterial pressure  
 1284 arterial pressure  
 1285 arterial pressure  
 1286 arterial pressure  
 1287 arterial pressure  
 1288 arterial pressure  
 1289 arterial pressure  
 1290 arterial pressure  
 1291 arterial pressure  
 1292 arterial pressure  
 1293 arterial pressure  
 1294 arterial pressure  
 1295 arterial pressure  
 1296 arterial pressure  
 1297 arterial pressure  
 1298 arterial pressure  
 1299 arterial pressure  
 1300 arterial pressure

% of dose/g blood

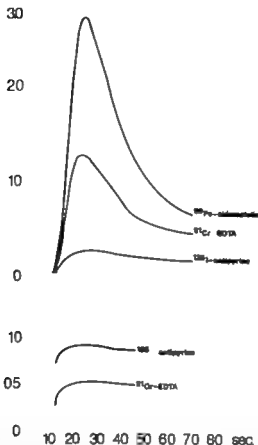


Fig. 2. Results of single injection of 1 ml plasma containing  $^{59}\text{Fe}$ -albumin,  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine. Various concentration curves (above) per cent of injected amount of isotope per gram blood in each sample. The injection was made at time zero. From the data obtained for each blood sample, extraction values for the two diffusible indicators were calculated (below). In this experiment plateau levels for extraction were achieved very late in the upstroke curve for the non-diffusible indicator. Note that initial extraction values are lower than the plateau values.

### Presentation of the relations between extraction (E) blood flow (Q) and permeability-surface area product (PS)

The main purpose of the present investigation was to study blood tissue exchange by the indicator dilution method in some experimental situations where arterial or venous blood pressure was changed or where capillary blood perfusion was changed as a consequence of shock or trauma. The experiments were not designed to yield any direct information about capillary permeability but rather to permit observation of PS changes, which, in relation to blood flow could possibly be used for comparison between different groups of experiments.

and convenient to plot extraction values (Cr-EDTA) around of theoretical PS curves showing the interrelationship of constant PS values in accordance with the equation seen in Fig. 3. In this way any observed change of extraction

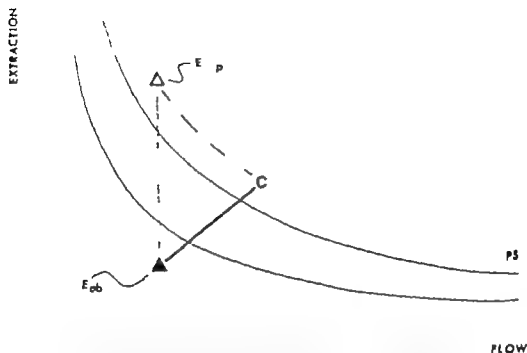


Fig. 3 Schematic drawing illustrating how a change in extraction from control (C) can be estimated in relation to various values of the PS product [background curves according to  $E = 1 - \exp(-PS/Q)$ ]. Mathematically this change may be expressed as a ratio between the extraction value obtained ( $E_{obs}$ ) and the extraction value expected to result from an unchanged value of PS and a similar change in flow ( $E_{exp}$ ).

could be visually compared to the one expected with the change of Q at constant PS. Each figure containing data from a group of experiments (Chapters 5-6-8), is accompanied by a figure illustrating mean changes (in per cent) in PS and blood flow for paired comparisons with the control set to 100 % for PS and blood flow.

The extraction of antipyrine is only presented in the tables since as will be shown the flow dependence of extraction was small or non-existing within the flow range studied.

## Statistical methods

The objectives of the statistical analysis of antipyrine blood flow (Cr-EDTA). The PS values of

for iodide Cr-EDTA and (iodide Cr-EDTA)

for iodide Cr-EDTA and (iodide Cr-EDTA) and flow (Q)

and extraction (E) as

$$PS = -Q \ln(1 - E)$$

where  $\ln$  denotes the natural logarithm.

These parameters and their interrelationships were studied separately by a computerized statistical analysis comprising data from the control state and they were also analysed in relation to some variables, which may be applicable in the assessment of the condition of the experimental animal (see Chapter 4).

To evaluate the response in the various parameters following arterial hypotension hemorrhage etc. mean values and the limits for the mean plus and minus one standard deviation were computed for all available pairs of observations. The changes were also calculated in per cent (except for antipyrine extraction), giving mean values and a 99 % confidence limit. The change is considered to be statistically significant at the 1 % level if both confidence limits are either less (decrease) or larger (increase) than 100 %. For antipyrine extraction, changes are statistically significant if both limits are either positive or negative



## ANALYSIS OF VENO-CONCENTRATION AND EXTRACTION CURVES (INDICATOR DILUTION METHOD)

Since the indicator dilution method has been employed throughout the present studies, it was considered to be of interest to present some complete curves, illustrating this method at different blood flow levels and, in turn, with different Cr-EDTA extractions at these flow levels. Each  $E/Q$  value of the diffusible indicators (Cr-EDTA, iodide and I-antipyrine) presented in the following chapters was estimated from one or two (in control and after hemodilution) sets of such curves. Each set of curves represents a single injection of isotopes and the serial blood samples which were subsequently collected (Fig. 2).

### Results

There was a considerable variation of the configuration of veno-concentration curves. The time for blood sampling after each intra-arterial injection of indicators was adjusted to a length appropriate for prevailing blood flow rate. At very low blood flows, concentration curves were occasionally obtained where peak concentration of the reference indicator was not reached within the sampling time. Only very rarely was blood sampling, at relatively high blood flows, terminated before peak concentration had been reached. The configuration of the veno-concentration curves, however, was not only depending on the blood flow rate.

Within the low blood flow range, curves were obtained, which displayed an individual variability. This is exemplified in Fig. 4 where the curves represent three different measurements from two experiments. In the upper part of the graphs, the appearance time is seen to vary by 10 seconds and the time to reach peak concentration from very short time to more than 15 seconds despite comparable values of total blood flow. The corresponding extraction curves (lower part of graphs) display a plateau of extraction values, which corresponds to parts of the veno-concentration curves before or around the peak. The  $E_{Cr}$  values vary between .50 and .13. Also at comparable, higher blood flows, variations of time-concentration curve configuration and extraction levels were found (Fig. 5).

### Comments

It was often found that time-concentration curves had not been recorded long enough to display a curve peak or a down-slope. Nevertheless, extraction values forming a plateau were obtained from the existing part of the curve. Attempts to measure the blood flow by calculation of the area under the curve after extrapolation from the early down-slope of the curve (for indiffusible tracer) were not made due to the scarcity of adequate



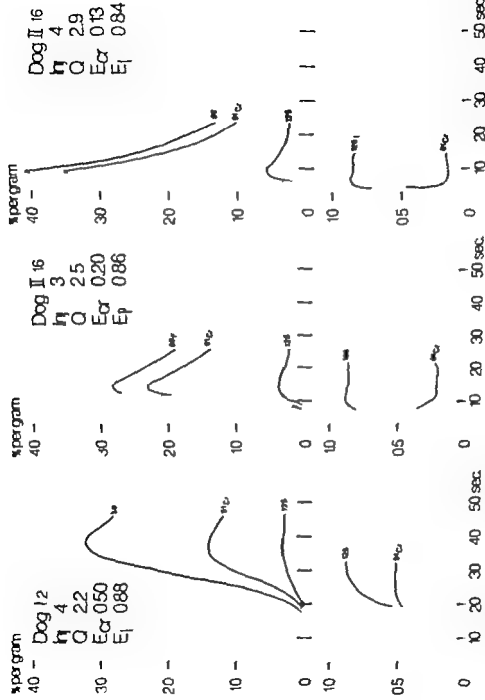


Fig. 4 Various concentration (or indicator dilution) curves for  $^{59}\text{Fe}$ -EDTA and I-antipyrine (above). Corresponding extraction curves (for C-EDTA and I-antipyrine) are seen below each set of dilution curves. Data are presented in accordance with the description given in Fig. 2. The two sets of curves (the left refer to an experiment on the effects of regional arterial hypotension; the others to measurements made in period of normotensive hypotension). The curves have been selected in order to illustrate the variation in the shape of curves obtained while ranges of comparably low blood flow levels. The extraction values for C-EDTA in sec. to vary 1 large extent.

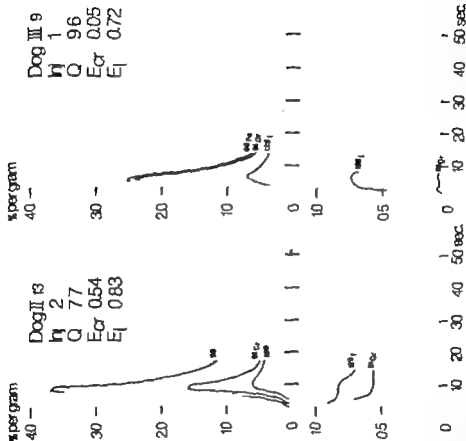


Fig. 3 Lead detector dilution curves for  $^{203}\text{Pb}$ -antipyrine,  $^{65}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine (above). Extraction curves, for Cr-EDTA and I-antipyrine, calculated from the data above, are seen below each set of venous concentration curves. As in Fig. 4 graphs have been selected so as to illustrate the variations in the configuration of curves obtained, in this case 1 comparable, higher rates of blood flow  $N$  is the different plateau levels for the extraction of Cr-EDTA.

curves. For the same reason mean transit time was not calculated, the error introduced by extrapolation even in more "complete" curves was reckoned to be too large (cf. Rippa and Stage 1978)

## Summary

Some indicator dilution curves have been presented representing three isotopes at different rates of blood flow. The curves illustrate

- 1) that the  $^{125}\text{I}$ -antipyrine extraction was approx. 0.8 and independent of blood flow rate and of the  $^{51}\text{Cr}$ -EDTA extraction,
- 2) that, at two different levels of perfusion rate (2 - 3 and 8 - 10 ml/min/100 g resp.),  $^{51}\text{Cr}$ -EDTA extraction might vary considerably at different injections,
- 3) that for each isotope there was a plateau of consecutive extraction values soon after the appearance of the isotope in the venous effluent. The level of this plateau was used as extraction value for the indicator

In addition it was concluded

- 4) that the configuration of the dilution curves did not permit any regular estimation of blood flow (from the dilution curve of the indiffusible indicator). The limited sampling time made available curves inadequate for estimation of mean transit time

## DESCRIPTION OF THE CONTROL STATE (INDICATOR DILUTION METHOD)

The variability described in the previous chapter is amply illustrated in the series of  $E_{Cr}/Q$  diagrams which are to be presented in the following chapters. It was seen both during the control state and during the activated states. Due to the variability of  $E/Q$  a separate analysis of the control state was considered to be of interest.

In order to ascertain whether any justification could be found to divide the group of control data, e.g. according to level of PS ( $Cr-EDTA$ ), the relations between the parameters and different background variables concerning the condition of the experimental animal were statistically analysed. It could be argued that some animals had, in terms of reaction to preparation trauma, suffered a more profound disturbance of their peripheral circulation than others (Appelgren 1972). If this were to be the case, the mode of reaction to different experimental procedures after the control period may possibly vary accordingly.

## Methods

The control values for extraction ( $E$ ) of the diffusible, water-soluble indicator (usually  $^{51}Cr-EDTA$ ) extraction of  $^{125}I$ -antipyrine, PS ( $^{51}Cr-EDTA$ ) and blood flow ( $Q$ ) were subjected to a computerized statistical analysis in order to describe the control state in 63 dogs. In each experiment one or two control measurements were used in form of their mean value. The measurements (in Chapters 5–9) of  $Q$  and of  $E$  and PS for the lipid insoluble indicators (iodide ion,  $^{51}Cr-EDTA$ ) were subjected to a logarithmic transformation since their distributions were not normal but positively skewed. The means presented are the geometric means and the description, in the tables, of the dispersion in terms of the mean plus and minus one standard deviation is not symmetric around the mean. For the  $I$ -antipyrine measurements only  $E$  is considered and these measurements have not been transformed.

## Results

A large scattering of the  $Q$ ,  $E_{Cr}$  and consequently  $PS_{Cr}$  values was found, mainly reflecting an individual variability of the vascular bed in the participating animals. The distributions of  $Q$ ,  $PS_{Cr}$  and  $E_{Ap}$  are presented in Fig. 6. The mean values were within the range to be expected in a resting musculature:  $Q = 4.6$  ml/min/100 g (MV + SD 6.9, MV - SD 3.1),  $E_{Cr} = 0.20$  (MV + SD 0.44, MV - SD 0.09) and  $PS_{Cr} = 1.15$  ml/min/100 g (MV + SD 2.68, MV - SD 0.49). The mean  $E_{Ap}$  was .79 (SD .08). Some relations between the

parameters were evaluated

- 1)  $E_{Cr}$  vs Q correlation  $-.29$  (rank correlation  $-.33$ )
- 2)  $\ln(1-E_{Cr})$  vs  $1/Q$  correlation  $-.20$  (rank correlation  $-.33$ )
- 3)  $PS_{Cr}$  vs Q correlation  $.25$  (rank correlation  $.26$ )
- 4)  $E_{Ap}$  vs Q correlation  $.21$

The first two of these four correlations were statistically significant.

No statistical justification could be found to treat the group of control data as more than one homogeneous set of values. No significant correlations were found to exist between any of the parameters (Q E PS) and the following variables: body weight, rectal temperature, arterial blood pressure, hematocrit and preparation time.

**Comment.** The use of the control state for the evaluation of the activated state

To illustrate the response produced by regional arterial hypotension, hemorrhage and hemodilution, the data are presented in

- a) tables showing mean values and limits for the mean, and
- b) tables showing mean changes (in per cent) and their confidence limits for paired comparison of measured values for  $PS_{Cr}$ -EDTA and Q in the control state (most often mean of two values) and corresponding PS/Q pairs in the activated state.

For the same indicators (iodide ions  $^{51}Cr$ -EDTA) changes in per cent are also presented for observed extraction in relation to expected extraction at unchanged PS (with identical change of flow) expressed as their ratio (Fig. 3).

This parameter  $E_{OBS}/E_{EXP} \times 100$  should give results very similar to those obtained for the change in PS, at least at low extractions. In any comparison all available pairs of observations have been used.

Changes of extraction for the lipid soluble indicator 1-antipyrine are expressed as absolute differences, considering the small and flow independent changes from the control to the activated state.

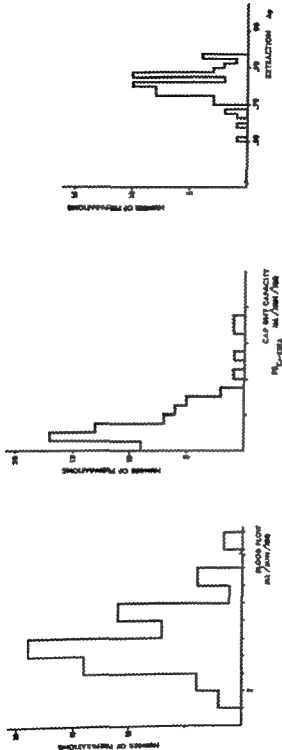


Fig. 6. Histograms of values for blood flow, capillary diffusion capacity (PSC - EDTA) and extraction of t-antipyrine. The determinations were made here there were no obvious indications of any induced injury i.e. the measurements were all made in the control state. It can readily be seen that the values for PSC - EDTA have a skewed distribution, which is also the case for the distribution of blood flow. The skewed values of blood flow and PSC - EDTA, given in the text with asymmetric standard deviations, are due to the skewed distributions.

trated in Fig. 7 (right panel).

The mean change of  $^{125}\text{I}$ -antipyrine extraction was 0.03 ( $-0.03$  0.09).

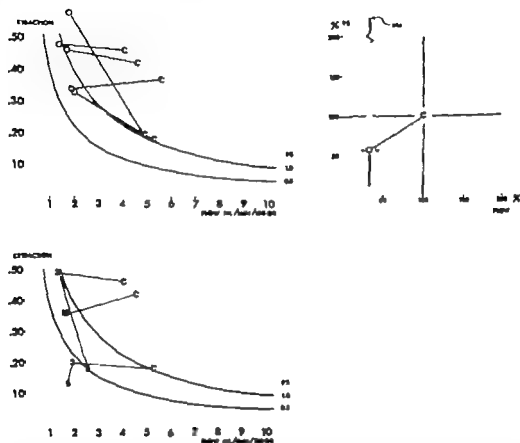


Fig. 7 Single-injection multiple-indicator dilution method. Extraction/flow diagrams to the left, changes in PS and flow (in per cent) to the right. The changes in extraction and PS for Cr-EDTA, induced by short-term regional arterial hypotension in five experiments (above left). An open circle indicates the individual values in relation to its control (C).

To the right, the changes in PS and flow [paired comparisons with control (C)] have been expressed in per cent with the control value set to 100 %. The open circle indicates mean values of PS and Q; 99 % confidence limits for PS and Q have been drawn. At reduced perfusion pressure (approx 50 mm Hg) both blood flow and PS are seen to decrease. The change of PS, however, is statistically insignificant (upper right).

In three of the experiments (below), extraction values were also obtained after three (3) and five (5) hours during a period of prolonged hypotension.

	Control			Hypotension		
	N	Mean	Mean $\pm$ SD	N	Mean	Mean $\pm$ SD
<i><sup>51</sup>Cr-EDTA</i>						
Blood flow	5	5.4	4.3-6.7	5	1.7	1.5-2.0
Extraction	5	0.30	0.19-0.47	5	0.43	0.34-0.54
PS	5	1.74	0.95-3.17	5	0.99	0.75-1.31
<i><sup>125</sup>I-antipyrine</i>						
Extraction	5	0.84	0.80-0.88	5	0.87	0.82-0.92

Table I. Mean blood flow, extraction and permeability surface area values before and after reduction of perfusion pressure.

	Effect %	Conf. limits %
<i><sup>51</sup>Cr-EDTA</i>		
Blood flow	33	(24-45)
Extraction	142	(51-399)
PS	57	(11-792)
Observed/expected extraction	61	(31-121)
<i><sup>125</sup>I-antipyrine</i>		
Extraction (absolute difference)	0.03	(-0.03-0.09)

Table II. Change of extraction, blood flow and permeability surface area following reduction of perfusion pressure.



## Comments on results

In the present study PS (Cr-EDTA) and blood flow were seen to decrease ~~in~~ decreased perfusion pressure. Due to the scarcity of data, no statistical analysis could be made ~~to~~ differentiate between the effects of the short-term and the prolonged moderate reduction of perfusion pressure. It might be argued that a PS decrease would have been demonstrated with statistical significance in the present study if all data had represented a prolonged period of hypotension. Judging from the few available measurements (see Fig. 7 below left) a tendency may be present to support this reasoning but a more extensive study would be necessary to justify a more conclusive comment.

## Summary

The present data indicate a reduction of the exchange of small molecules in the dog's skeletal muscle during regional arterial hypotension (40 – 60 mm Hg). Compared to control values, the blood flow was reduced to 33 % and the PS product for  $^{51}\text{Cr}$ -EDTA was reduced to 57 %. The change of  $^{125}\text{I}$ -antipyrine extraction was insignificant. Data were too few to permit any statistical analysis to compare the effects of short-term and prolonged regional arterial hypotension.

## BLOOD TISSUE EXCHANGE DURING HEMORRHAGIC HYPOTENSION

In two previous preliminary reports data were presented from studies on blood tissue exchange of small molecules in hemorrhagic shock by the indicator dilution method (Dahlberg and Lewis 1973 1976). In one of these studies (1976), a local clearance method was simultaneously employed. The results from the two reports are here analysed as a single group of data and with the same statistical approach as was used elsewhere in this monograph.

## Experimental procedure

Hemorrhagic hypotension was produced by a rapid reduction of the blood volume decreasing the arterial blood pressure to about 50 mm Hg. By additional bleedings blood pressure was maintained between 40 and 60 mm Hg.

Blood tissue exchange was studied by the indicator dilution method and, in some experiments, simultaneously by the local clearance method. For the former method, different combinations of indicators were used in addition to  $^{59}\text{Fe}$ -sclerophilin which acts as an indiffusible vascular marker (see Table III). In 12 experiments  $^{51}\text{Cr}$ -EDTA was paired with  $^{125}\text{I}$ -antipyrine in seven, iodide ion was used instead of  $^{51}\text{Cr}$ -EDTA ( $^{131}\text{I}$ , 5 experiments) or  $^{125}\text{I}$ -antipyrine ( $^{125}\text{I}$  2 experiments). In two experiments  $^{125}\text{I}$ -RIHSA was used instead of  $^{125}\text{I}$ -antipyrine (but together with  $^{59}\text{Fe}$ -sclerophilin).

Number of experiments	$^{51}\text{Cr}$ -EDTA	$^{131}\text{I}$	$^{125}\text{I}$	$^{125}\text{I}$ -RIHSA	$^{125}\text{I}$ -antipyrine
5		*			*
2	*		*		
2	*			*	
12	*				*

Table III. Combinations of indicators used in addition to vascular marker for the indicator dilution method in experiments on hemorrhagic hypotension.

In the local clearance method the transport of  $^{51}\text{Cr}$ -EDTA was studied by external detection following the intramuscular injection of this isotope mixed with  $^{133}\text{Xe}$ . The ratio of their clearance values gives  $^{51}\text{Cr}$ -EDTA extraction if  $^{133}\text{Xe}$  clearance equals blood flow. The technique was employed in ten experiments. For each set of measurements, tissue-to-blood transport was measured twice before and twice after two measurements of blood to-tissue transport. Measurements were carried out, apart from control state, at two, four and six hours after the onset of the bleeding.

## Results

Values for iodide ion ( $^{131}\text{I}$  or  $^{125}\text{I}$ ),  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine from measurements by the indicator dilution method are presented in Table IV. Mean values and the limits for the mean plus and minus one standard deviation are given for measurements before and after hemorrhage.

	Control			2 hrs hemorrhage			4 hrs hemorrhage			6 hrs hemorrhage		
	N	Mean	Mean $\pm$ SD	N	Mean	Mean $\pm$ SD	N	Mean	Mean $\pm$ SD	N	Mean	Mean $\pm$ SD
<i>Iodide ion (<math>^{131}\text{I}</math>, <math>^{125}\text{I}</math>)</i>												
Blood flow	7	3.9	2.6-5.8	7	1.5	1.1-2.1	5	1.4	0.6-2.2			
Extraction	7	0.36	0.29-0.46	7	0.48	0.38-0.60	5	0.52	0.43-0.63			
PS	7	1.77	1.11-2.82	7	0.99	0.57-1.73	5	1.01	0.77-1.32			
<i><math>^{51}\text{Cr}</math>-EDTA</i>												
Blood flow	16	3.1	3.0-8.8	14	1.8	0.8-3.7	12	1.6	0.7-4.2	7	1.3	0.6-2.8
Blood flow LCM	9	7.3	4.6-12.0	9	4.3	2.6-6.9						
Extraction	16	0.15	0.07-0.33	13	0.23	0.12-0.43	12	0.32	0.15-0.55	5	0.24	0.16-0.36
PS	16	0.85	0.31-2.31	13	0.52	0.17-1.58	12	0.47	0.16-1.35	5	0.25	0.18-0.66
PS, LCM	9	0.94	0.73-1.23	9	0.49	0.34-0.71						
<i><math>^{125}\text{I}</math>-antipyrine</i>												
Extraction	17	0.79	0.71-0.87	13	0.87	0.83-0.91	11	0.88	0.84-0.92	4	0.92	0.90-0.94

Table IV. Mean blood flow, extraction and permeability surface area values before and after hemorrhage. LCM indicates local clearance method.

Extraction values for  $^{51}\text{Cr}$ -EDTA are only given for the indicator dilution method.

The effect of hemorrhage is presented in Table V. Any change for the lipid-insoluble indicators is given as the mean effect and a 99 % confidence interval. The results obtain

	Duration of hemorrhagic hypotension					
	2 hours		4 hours		6 hours	
	Effect %	Conf limits %	Effect %	Conf limits %	Effect %	Conf limits %
<i>Iodide ion (<sup>131</sup>I, <sup>125</sup>I)</i>						
Blood flow	39	(21-72)	35	(11-119)		
Extraction	130	(87-176)	153	(71-242)		
PS	56	(26-118)	64	(23-116)		
Observed/theoretical extraction	70	(43-113)	78	(56-103)		
<i><sup>51</sup>Cr-EDTA</i>						
Blood flow	34	(22-54)	31	(18-54)	25	(12-52)
Blood flow LCM	58	(49-69)				
Extraction	137	(97-192)	201	(125-325)	164	(55-485)
PS	30	(25-97)	39	(27-131)	54	(10-279)
PS, LCM	51	(28-89)				
Observed/theoretical extraction	88	(35-95)	70	(41-120)	54	(17-171)
<i><sup>125</sup>I-antipyrine</i>						
Extraction (absolute difference)	0.06	0.01-0.11	0.08	0.02-0.14	0.09	0.01-0.19

Table V. Change of extraction, blood flow and permeability surface area following hemorrhage. LCM indicates local clearance method. The change in the extraction of <sup>51</sup>Cr-EDTA is only given for the indicator dilution method.

ed by the indicator dilution method are also presented in Figs. 8 and 9. Fig. 8 shows, to the left, the individual changes of iodide ion extraction and blood flow for each animal against a background of theoretical PS lines, after two and four hours of hemorrhagic hypotension. Fig. 9 shows the corresponding data for <sup>51</sup>Cr-EDTA after two, four and six hours of hemorrhagic hypotension. In both figures, to the right, the changes in blood flow and PS (in per cent of control) are presented in accordance with Table V. The largest PS reduction, 50 % for <sup>51</sup>Cr-EDTA after a two hour period of hemorrhagic hypotension, is statistically significant.

In the two experiments where <sup>125</sup>I-RHSA was studied, this substance was found to behave in the same manner as <sup>59</sup>Fe-aiderophillin.

<sup>125</sup>I-antipyrine extraction was found to increase slightly after bleeding. The increase, however, was not statistically significant.

The results obtained by the local clearance method are also included in Tables IV and V. Fig. 10 shows all values (left panel) for Cr-EDTA extraction and blood flow (Xenon clearance) and also (right panel) the changes in blood flow and PS after two hours (in accordance with Table V). The PS reduction after two hours of hemorrhagic hypotension, to 51 %, is statistically significant.

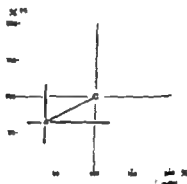
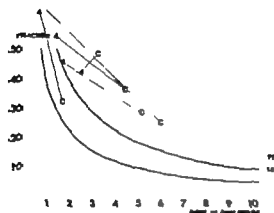
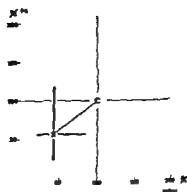
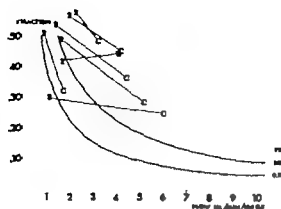


Fig. 8 Single-injection multiple-indicator dilution method. Extraction changes for iodide ion induced by hemorrhagic hypotension (left in figure). Data at two (2) hours (above) and at four (4) hours (below) after the onset of rapid bleeding. Perfusion pressure approx. 50 mm Hg.

Changes in blood flow and PS (paired comparisons) in per cent (right). Mean values of PS and Q with 95% confidence limits are given.

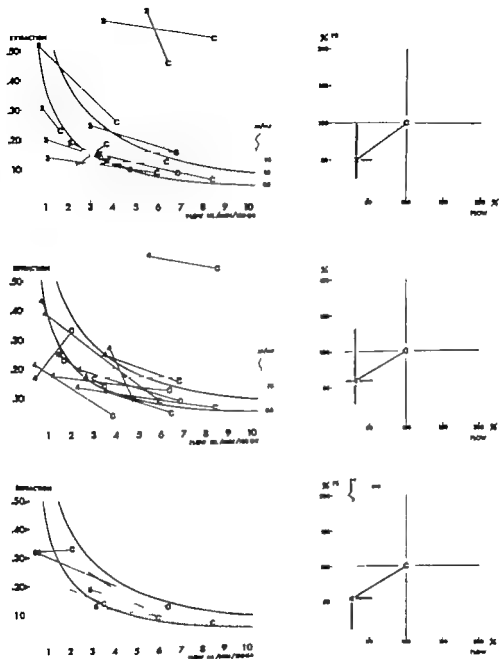


Fig. 9 Single-injection multiple-indicator dilution method. Changes in extraction and PS for Cr-EDTA at two (2) four (4) and six (6) hours during *Acromegalic hypotension* (perfusion pressure approx. 50 mm Hg).

The decrease in PS is statistically significant (both confidence limits less than 100%) at two (2) hours after the onset of bleeding. The number of surviving animals is gradually reduced during the shock period.

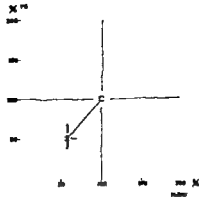
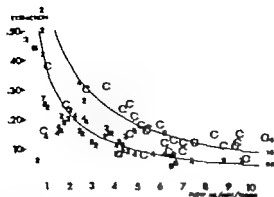


Fig. 10. Local clearance method. Extraction/flow diagram to the left, changes in PS and flow (in per cent) to the right.

Changes in extraction of Cr-EDTA during *Hemorrhagic Hypotension* compiled data from ten experiments where the method has been employed simultaneously with the indicator dilution method. Measurements were made in the control state (C) and at various times during the shock period, two (2) - seven (7) hours after the onset of bleeding.

The changes in PS and blood flow in per cent (right) indicate that the decrease in the values for these parameters (paired comparisons) is statistically significant after two (2) hours of hypotension (cf. data for C-EDTA obtained by indicator dilution method Fig. 9).

## Comments on results

The extraction values of iodide were found to be slightly higher than those of Cr-EDTA. This was expected to occur as a consequence of the different molecular dimensions of the two substances. During the course of the hemorrhagic shock there was an increasing number of animal deaths and the scattering of PS values was therefore seen to increase.

## Summary

During hemorrhagic shock, the blood tissue exchange in the dog's skeletal muscle was found to decrease. The transport of small lipid insoluble molecules between the blood and the interstitial fluid diminished markedly: a 50 % reduction of the capillary diffusion capacity (PS) was estimated both by the indicator dilution method and by the local clearance method.

## BLOOD FLOW AND BLOOD TISSUE EXCHANGE IN TRAUMATIZED SKELETAL MUSCLE

In the present chapter data are presented which were obtained in studying the blood tissue exchange in injured skeletal muscle by the indicator dilution method. The hind leg musculature of the dog was traumatized by a series of contusions over a wide area. It was attempted to find out

- 1) If injury produces, consistently an initial increase in blood flow
- 2) How the PS response is related to the response in blood flow

### Experimental procedure

The anaesthetized dogs (10) were ventilated with intermittent positive pressure ventilation. After control measurements (first and second isotope injection = C) injury to the dog's left hind leg was inflicted by delivering 200 heavy strokes from a blunt wooden instrument. Following this, one injection (three isotopes in plasma) was made as soon as possible within the first few minutes (D), a second 10 minutes (E), a third 20 minutes (F) and a fourth 60 minutes (G) after the contusion.

### Results

All animals survived the whole period of observation. In one experiment, all extraction values, both during the control state and after the trauma were negative probably as a result of a technical error. In this animal, there was a temporary blood flow increase following the trauma (not illustrated). Fig. 11 (upper panel) shows the blood flow rates for each experiment. The double vertical line indicates trauma. In four of these experiments a temporary blood flow increase was seen to follow the injury giving, in total a flow increase early after trauma in five of the ten dogs. In five experiments a blood flow decrease followed, only in two of these the blood flow tended to return towards control level. The dogs that responded to injury with a blood flow increase displayed a blood pressure reduction that was less marked than the one shown initially by the others. In the two dogs that tended to improve by increased blood flow after an initial decrease, a parallel blood pressure restoration was observed. The level of blood flow or arterial pressure at control was not related to the blood flow response to the injury.

Fig. 11 (lower panel) also shows the PS response to injury in each experiment. A PS increase was only seen in one experiment, where a single control value represented a relatively low PS value at low blood flow (III:6). Three dogs showed a prolonged PS decrease despite initial increase in blood flow. If blood flow decreased, PS always decreased. Thus, PS increase was rarely seen, whereas blood flow increase was seen in half of the experiments.



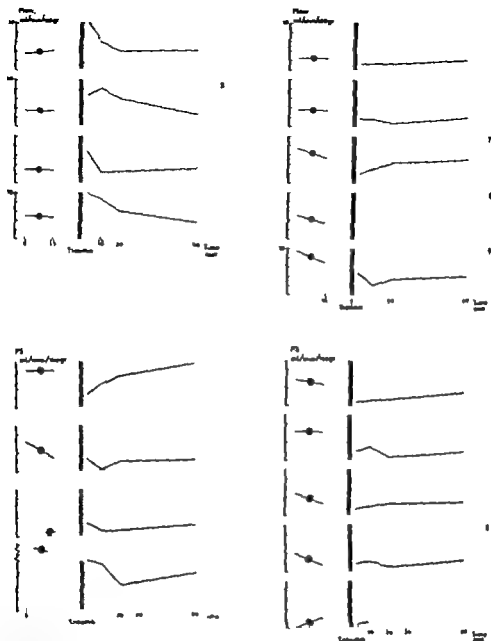


Fig. 11 Response in blood flow and PS (Cr-EDTA) to injury (single-injection multiple-indicator dilution method)

The blood flow (upper graphs) was measured immediately after the trauma and also after 10, 20 and 30 minutes. The mean control value is indicated by a closed circle. In four experiments there was an increase in blood flow (left) maintained for varying period of time. In five experiments, the blood flow decreased after injury (right).

The response to PS is seen in the lower graphs. With one exception (experiment 6) PS was reduced after injury

Fig. 12 shows individual extraction values for  $^{51}\text{Cr}$ -EDTA at the actual blood flows before (C) immediately following (D) and 10, 20 and 60 minutes after trauma (E, F and G). There is a separate diagram for each dog.

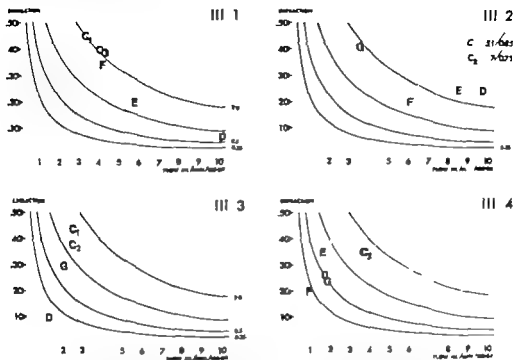


Fig. 12a. Response to injury. Extraction/flow values ( $\text{Cr}$ -EDTA) obtained by the single-injection multiple-indicator dilution method. Control values are indicated by  $C_1$  and  $C_2$ . The values obtained after injury are indicated by D, E, F and G (<5, 10, 20 and 60 minutes). The curves are drawn of constant values of PS product shown in the right margin. In one of the experiments (III 2) indicating temporary increase in blood flow after injury the control values for extraction of  $\text{Cr}$ -EDTA are exceptionally high.

If the change of extraction values was analysed with respect to the expected change at constant PS a statistical analysis revealed an effect in accordance with Table VI.

Observed/expected extraction

D	E	F	G
57 %	57 %	63 %	80 %
(22-144) %	(19-171) %	(30-133) %	(45-140) %

Table VI. Change of the value for observed/expected extraction for  $^{51}\text{Cr}$ -EDTA induced by injury

D-G denote time after trauma. Immediately 10 20 and 60 minutes. The response was most pronounced initially in the period following injury. As stated earlier the response in PS should be similar (cf Fig. 13). The changes, however, were not uniform or statistically significant.

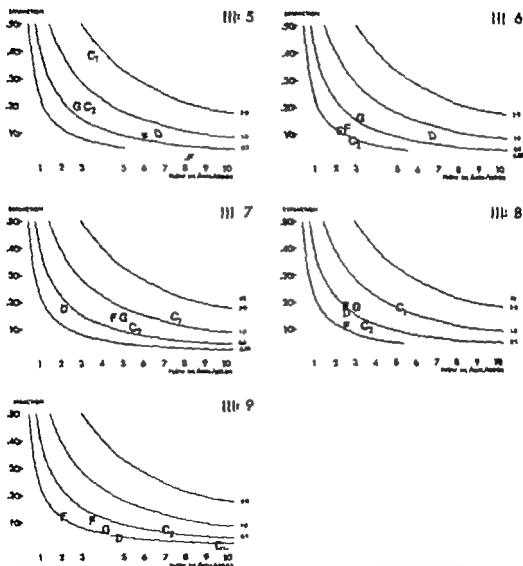


Fig. 12b. Response to injury. Extraction/flow values (Cr-EDTA). In one experiment (III: 6) only one value for Cr-EDTA extraction was obtained, representing a PS value for the control state that is remarkably low. Injury is seen to induce, in this experiment, an initial increase in PS.

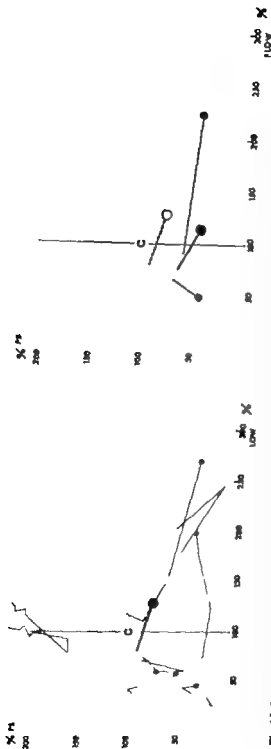


Fig. 13. Response to injury in the left graph, response in  $\text{P}^3\text{O}_2$ -EDTA and blood flow presented for each experiment. Changes are given in per cent of control values (C). Small filled circles represent first values obtained immediately after injury and then lines connect subsequent values after 10, 20 and 60 minutes. In all experiments except one,  $\text{P}^3$  is decreased in comparison with control. The larger filled circle with the thick line indicates the mean effect of injury in the above experiments and represents the values obtained immediately after injury (filled circle) and after 60 minutes (end point of line). If the only experiment with an increase in  $\text{P}^3$  is excluded this symbol is displaced towards lower values for  $\text{P}^3$  as illustrated in the right graph. The smaller filled circles (right graph) represent the mean effects of injury if the same data are divided according to initial response in blood flow.

In Fig. 13 (left graph) the response in PS and Q is presented for each experiment. The changes are given in per cent of the control values (C). Except in one experiment, PS was markedly reduced and the response in PS was seen to be unrelated to the change in blood flow. In the right part of the figure the mean effects are presented with respect to the different early response in blood flow i. e. flow increase or decrease after trauma. The decrease in PS was present within a few minutes after the period of traumatization. During the following hour there was a slight increase in PS towards control level and the blood flow rate was returning towards pre-trauma values.

$^{125}\text{I}$ -antipyrine extraction was unaffected by the injury. The mean, statistically insignificant, difference was 0.014 (S.E. 0.026).

### Comments on results

The response in PS for Cr-EDTA to injury seems to be independent of the change in total blood flow. This may indicate that the mechanisms which control the distribution of capillary blood flow are differently influenced by injury in comparison with those that govern the total blood flow.

### Summary

Blood flow and blood tissue exchange of  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine were studied in the dog's skeletal muscle before and after local injury. Following injury total blood flow immediately increased in one half of the experiments. In the other animals total blood flow decreased. PS was nearly always seen to decline after injury even when total blood flow increased.

## BLOOD FLOW AND BLOOD TISSUE EXCHANGE DURING NORMOVOLEMIC HEMODILUTION

In this chapter data are presented which were obtained in studying blood tissue exchange of  $^{51}\text{Cr}$ -EDTA by the indicator dilution method at various levels of normovolemic hemodilution. In some of the experiments the local clearance method was simultaneously used. The results have been analysed in accordance with the method used elsewhere in this monograph. The statistical analysis is different in comparison with a previous, preliminary report on some of the data (Dahlberg and Lewis 1975)

### Experimental procedure

The animals were initially splenectomized. Control measurements were made before the hemodilution started. For hemodilution, equal amounts of whole blood were exchanged for dextran 70 (Macrodextran, six per cent dextran in saline). Measurements were repeated at hematocrit levels of 25–30 % and 10–12 %. At each hematocrit level two measurements of tissue-to-blood extraction were made before and after a pair of measurements of blood to-tissue extraction.  $^{59}\text{FeCl}_3$ ,  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine were used as indicators for the indicator dilution method,  $^{51}\text{Cr}$ -EDTA and  $^{133}\text{Xenon}$  for the local clearance method.

### Results

Fifteen experiments were carried out. The indicator dilution method was used in all of these. Simultaneously the local clearance method was used in six experiments (in one of which the blood-to-tissue values had to be discarded because of a technical error). Due to hematocrit changes during hemodilution all flow rates are given as plasma flows and PS ( $\text{Cr}$ -EDTA) values are given in ml plasma/min/100 g tissue.

The mean values for flow  $^{51}\text{Cr}$ -EDTA extraction and PS for  $\text{Cr}$ -EDTA at control and after hemodilution are presented in Table VII.

The effect of hemodilution is presented in Table VIII. In Fig. 14 individual plasma flow changes and  $^{51}\text{Cr}$ -EDTA extraction changes (indicator dilution method) are shown at the two stages of hemodilution (left panels). Open circles represent values at hematocrit 25–30 % filled circles values at hematocrit 10–12 %. The changes in plasma flow and PS are also seen in Fig. 14 (right panels) in accordance with Table VII. A progressive increase in flow and PS is seen to follow the hemodilution.

Fig. 15 (left panel) shows the compiled data for tissue-to-blood  $^{51}\text{Cr}$ -EDTA extraction (local clearance method) at control (C) hematocrit 25–30 % (open circles) and hematocrit 10–12 % (filled circles). From the figure it appears that PS changes mean-

	Control			Level 1			Level 2		
	N	Mean	Mean $\pm$ SD	N	Mean	Mean $\pm$ SD	N	Mean	Mean $\pm$ SD
<i><sup>51</sup>Cr-EDTA</i>									
Plasma flow	14	2.2	1.6-3.1	14	3.4	2.4-4.8	13	4.5	3.0-6.7
Plasma flow LCM	6	3.8	2.0-7.0	6	5.9	2.6-13.7	6	7.5	3.8-15.1
Extraction	14	0.23	0.10-0.52	14	0.25	0.13-0.48	13	0.23	0.14-0.38
PS	14	0.62	0.23-1.62	14	1.02	0.52-2.00	13	1.31	0.76-2.28
PS LCM	6	0.65	0.50-0.85	6	0.74	0.62-0.88	6	0.57	0.43-0.76
<i><sup>125</sup>I-antipyrine</i>									
Extraction	14	0.80	0.70-0.90	14	0.77	0.67-0.87	13	0.85	0.80-0.90

Table VII. Mean plasma flow extraction and permeability surface area before and after normovolemic haemodilution. LCM indicates local clearance method. Extraction values for  $^{51}\text{Cr}$  EDTA are also given for the indicator dilution method.

	Level of hemodilution			
	Effect <sup>1</sup> %	Conf. limits %	Effect <sup>2</sup> %	Conf. limits %
<i>51Cr-EDTA</i>				
Plasma flow	153	(127-184)	215	(167-276)
Plasma flow LCM	156	(101-239)	198	(126-311)
Extraction	112	(82-156)	107	(64-177)
PS	165	(105-259)	222	(137-358)
PS LCM	113	(87-147)	88	(51-151)
Observed/expected extraction	165	(113-243)	210	(119-370)
<i>125I-antipyrine</i>				
Extraction (absolute difference)	-0.01	(-0.07-0.05)	0.07	(0.01-0.13)

Table VIII. Change of plasma flow, extraction and permeability surface area following normovolemic hemodilution. LCM indicates local clearance method. The change in the extraction of  $^{51}\text{Cr-EDTA}$  is only given for the indicator dilution method.



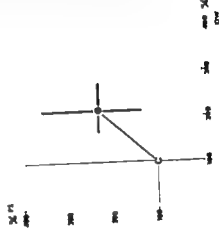
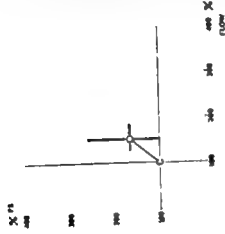
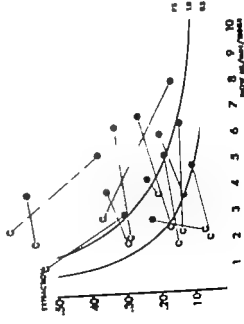
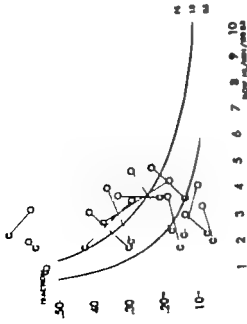


Fig. 14 Effects of normo-  
polyemic haemodilution. Single-in-  
jection multiple-indicator dilution  
method (14 experiments).  
Extraction (Cr-EDTA)/plasma  
flow diagrams to the left,  
changes in PS (Cr-EDTA) and  
plasma flow (as per cent) to the  
right.

The upper graphs represent the  
first level of haemodilution (he-  
matocrit 25–30%). Values ob-  
tained (indicated by open circles)  
in relation to control values (C).

The lower graphs represent the  
second level of haemodilution  
(hematocrit 10–12%). Values  
obtained (indicated by filled  
circles) in relation to control  
values (C).

The changes in PS (Cr-EDTA)  
and plasma flow are statistically  
significant at the two levels of  
haemodilution. Note the progres-  
sive increase in both plasma flow  
and PS (Cr-EDTA).

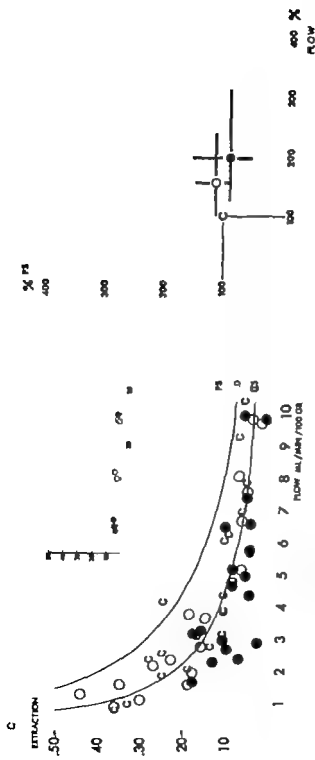


Fig. 13 Effects of normovolemic hemodilution. Local clearance method (used simultaneously with the indicator dilution method; 6 experiments). The left, extraction (Cr-EDTA)/plasma flow diagram for the compiled data. Open and filled circles represent values obtained at two levels of hemodilution (hematocrits 25 - 30 % and 10 - 12 %). The plasma flow values (X-ray clearance), including those for the control state (C) are spread over a wide range (cf. Fig. 14 indicator dilution method).

The right, the mean values for the changes in PS and plasma flow (paired comparisons with control). The 99 % confidence limits indicate that the increase in plasma flow is statistically significant at both hemodilution levels, whereas the changes in PS (Cr-EDTA) are negligible (in comparison with Fig. 14 indicator dilution method) and statistically insignificant. Note that the confidence limits for plasma flow are larger than those obtained by the indicator dilution method (Fig. 14).

by the local clearance method following hemodilution are small or none as compared to PS changes measured by the indicator dilution method. A statistical analysis supports this impression. Mean values are seen in Table VII the changes in Table VIII and in Fig. 15 (right panel).

The change in  $^{125}\text{I}$ -antipyrine extraction following hemodilution was statistically insignificant.

### Comments on results

Since the indicators are transported in the plasma, the PS (Cr EDTA) values are based on plasma flow rates rather than rates of whole blood flow. During hemodilution with a varying content of plasma in the blood, the change of plasma flow is therefore more relevant than the change of blood flow when considering substances like  $^{51}\text{Cr}$ -EDTA which are transported exclusively in plasma. PS expressed in this way is independent of hematocrit if measured under identical conditions, i.e. in a vasodilated vascular bed at high flows (Appelgren 1972b).

### Summary

Capillary transport of  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine was studied in the dog's skeletal muscle during graded, normovolemic hemodilution. The indicator dilution method was employed in all experiments, in some of the experiments the local clearance method was simultaneously used. The plasma flow and PS for  $^{51}\text{Cr}$ -EDTA were found to increase with hemodilution when measured by the indicator dilution method. The results obtained by the local clearance method confirmed the increase in plasma flow but no change in PS was seen when measured with this method.

## INFLUENCE OF INCREASED VENOUS PRESSURE ON TOTAL BLOOD FLOW AND BLOOD TISSUE EXCHANGE IN THE DOG HIND LEG

In the previous chapters, data have been presented from studies dealing with the blood tissue exchange of small molecules in the musculature of the dog's hind leg. The data represent both the control state and several activated states such as regionally decreased perfusion pressure, hemorrhagic hypotension, blunt tissue trauma and hemodilution. It has been assumed, when the blood-to-tissue exchange was measured, that the total venous return in the iliac vein represents the total blood flow through the musculature and its capillaries.

Although it is generally assumed that convection exchange is of minor importance in the blood-to-tissue exchange of molecules as small as Cr-EDTA, it was acknowledged that an increase of the venous pressure, if large, could possibly influence the transport of this indicator.

In order to study how an increased venous pressure affects the blood flow and the blood-to-tissue exchange, experiments were carried out where the regional venous blood pressure in the femoral vein was elevated for varying periods of time.

### Experimental procedure

The preparation and analytical procedures have previously been described. Some experiments were made to study the effects of a repeated, short term increase of venous pressure. The venous pressure was then temporarily raised by elevating the free end of the T-tube leading from the iliac vein, 13–14 cm above the level of the heart during the blood sampling period. In some experiments the venous pressure was elevated to the same extent (10 mm Hg) for longer periods by a partial occlusion of the iliac vein proximally to the T-tube. The T-tube was then linked to a saline manometer, disconnected only when the T-tube was used (in elevated position) for blood sampling. Care was taken not to inject the indicators until a constant venous blood flow could be observed following the venous pressure elevation. The venous pressure was not increased for more than 3 minutes with intervals of at least 15 minutes between repeated measurements. When venous pressure was raised continuously for several hours, measurements were made during the second, third and fourth hour after the onset of pressure elevation. No direct adjustments of arterial blood pressure were made during the course of experiments.

When the venous pressure was elevated, the blood flow through the iliac vein was regularly found to be markedly reduced but the perfusion pressure only slightly decreased in comparison with control. This was the case regardless if venous pressure was elevated for a short or long period of time (Fig. 16).

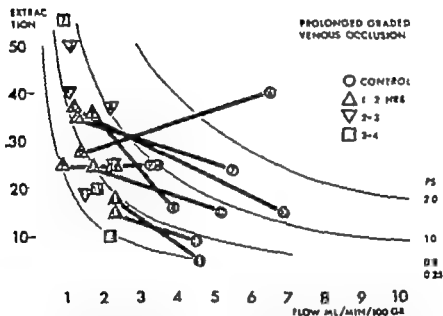
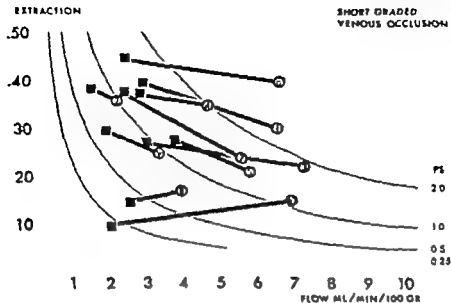


Fig. III Extraction (Cr EDTA) and blood flow values obtained after graded occlusion of the iliac vein in the non-injected hind limb of the dog. Blood flow was measured by timing the venous return in the iliac vein. The increase in venous pressure was 10 mm Hg. The numbers within the open symbols indicate the individual experiment. The open circles are the control values before the elevation of venous pressure.

The upper graph represents measurements made during periods of short-term elevation of venous pressure.

Values were also obtained (lower graph) during periods of prolonged elevation of venous pressure.

To clarify this finding, both arterial and venous blood flows were measured simultaneously both at normal and at increased venous pressure in a separate series of experiments. Arterial blood flow was measured by an electromagnetic flow-meter (femoral artery) and venous blood flow was measured by timing venous return. Three experiments were carried out for this purpose with the same basic surgical preparation as was used in the others. In one of these experiments, ligatures were initially inserted through the musculature as far proximal in the thigh as possible. When tightened around the muscles the ligatures excluded the main femoral vessels and the sciatic nerve so as to obstruct col lateral blood pathways.

$^{59}\text{FeCl}_3$ ,  $^{51}\text{Cr-EDTA}$  and  $^{125}\text{I-antipyrine}$  were used as indicators.

Nineteen experiments were carried out. In five experiments measurements were made during four repeated periods of the short term increase of venous pressure. From some of the other fourteen experiments, additional data were obtained soon after the venous pressure was elevated (five expts.) subsequent measurements in all the fourteen experiments were made to study the effect of a prolonged regional venous hypertension.

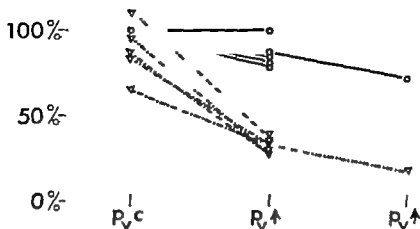
## Results

Fig. 17 shows results from the comparative measurements of arterial and venous blood flows. In the upper panel (A), blood flows are given in per cent of the arterial blood flow rate at control level of venous pressure ( $p_{vc}$ ). Five values were obtained after a short term increase of venous pressure (3 min.) and one value after a longer increase (30 min.). Of interest is the good agreement between arterial and venous blood flow during the control period. As venous pressure was raised (10 mm Hg) the arterial blood flow decreased to 90 % and venous blood flow to 1/3 of its control level. The perfusion pressure was reduced by 10 mm Hg from a control level of 100–120 mm Hg. After 30 minutes the blood flow had decreased further. In the lower panel of Fig. 17 (B) the blood flows are shown after ligation of the musculature (collateral blood flow). As venous pressure was raised, venous blood flow decreased at a much smaller degree and to the same extent as arterial blood flow.

In Fig. 16 the upper panel shows  $^{51}\text{Cr-EDTA}$  extraction values following a short term pressure increase the lower panel  $^{51}\text{Cr-EDTA}$  extraction values following a prolonged increase of venous pressure. Analysis of the venous blood flow rate shows a reduction to 54 % following the short-term increase in venous pressure. At continuous increase of venous pressure venous blood flow is reduced to 40 % after 1–2 hours and to 33 % after longer periods, both in relation to control flow. This is in fairly good agreement with the results shown in Fig. 17 (upper panel). The PS change was not statistically significant in any of the periods with increased venous pressure.

Fig. 18 shows the data from Fig. 16 following recalculation of venous blood flow values in accordance with the findings from the separate experiments (Fig. 17). With these cor

A



B

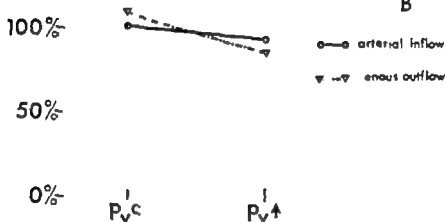


Fig. 17 Comparative measurements of arterial and venous blood flows at normal ( $p_{vc}$ ) and elevated ( $p_{v\uparrow}$ , arrow) venous pressure. Values obtained in the non-isolated hind limb preparation (graph A) and in the isolated preparation (graph B). Open circles indicate arterial blood flow measured by an electromagnetic flow probe on the femoral artery; triangles indicate blood flow measured by timing venous return from the iliac vein.

The arterial inflows before elevation of venous pressure ( $p_{vc}$ ) were set at 100%. The arterial blood flow values with increased venous pressure ( $p_{v\uparrow}$ , arrow) and all values for venous blood flow were expressed in per cent of the initial values for arterial blood flow.

Most measurements were made following control, during short-term elevation of venous pressure; the single values near the right margin of the upper graph represent values obtained after a 30 minute period of increased venous pressure.

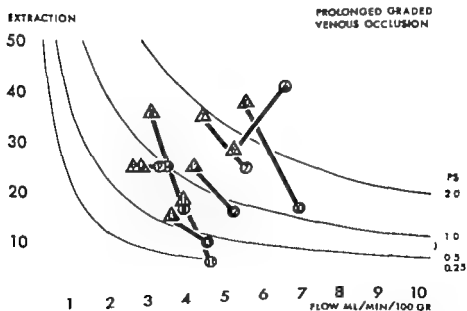
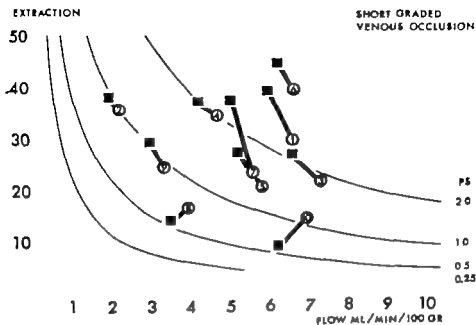


Fig. 18 Cf text in Fig. 16. The values for blood flow in the two diagrams have been recalculated according to the relation found between the arterial inflow and the registered venous outflow as illustrated in Fig. 17



rected' values for blood flow: the PS change, induced by the venous pressure decrease, remained practically unchanged both following short-term and prolonged pressure elevation.

$^{125}\text{I}$ -antipyrine extraction was not affected by any duration of venous pressure elevation, except after 1–2 hours of prolonged elevation when a small decrease was observed, mean difference 0.003 (SE 0.007).

### Comments on results

In the present study the total blood flow of the dog's hind leg was measured by obtaining the total venous return in the iliac vein. At control, this venous blood flow was found to be of the same magnitude as the arterial blood flow. When the venous pressure was raised, however, the venous blood flow decreased markedly. By measurement of the arterial blood flow it was found that the venous flow reduction equalled the increase of collateral venous return, i.e. the difference between the blood flow measured in the femoral artery and in the iliac vein. In contrast, the arterial blood flow through the hind leg decreased by only 10% when the venous blood pressure was raised (10 mm Hg). This blood flow reduction is consistent with the moderate reduction of perfusion pressure. The additional blood flow reduction, seen after a longer period of venous pressure elevation, is probably due to the gradual perfusion decline observed throughout the experiment.

The observed  $^{51}\text{Cr}$ -EDTA extraction values after venous pressure increase did not statistically differ from theoretically expected values at an unchanged PS. This was the case at any duration of venous pressure increase and regardless if the venous blood flow data were corrected or not with respect to the comparative calculations. It has been proposed that blood samples taken from the iliac vein are representative of blood from the entire capillary bed not only at control but also at increased venous pressure, when an increased collateral venous return is present.

### Summary

The blood-to-tissue transport was studied in the innervated dog hind limb at moderate to increased venous pressure (10 mm Hg). Multiple-indicator diffusion curves for  $^{59}\text{FeCl}_3$ ,  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine were obtained from the outflow through the iliac vein. The venous outflow was also used for measurement of the total blood flow. The total venous return in the iliac vein at normal venous pressure was found to be representative for the total blood flow in the hind leg.

It was found that the external pressure exerted on the iliac vein to raise the venous pressure caused a considerable diversion of the blood flow from the hind leg into collaterals. The transport of  $^{51}\text{Cr}$ -EDTA and  $^{125}\text{I}$ -antipyrine from blood to tissue was not changed by the elevation of the venous pressure.

## CHAPTER 10

### GENERAL DISCUSSION

#### A. Methodological considerations

Some of the difficulties concerning the theory behind the multiple indicator dilution method and the local clearance method are referred to model assumptions about capillary wall structure and hemodynamics and are made very evident when a mathematical relationship between such parameters as blood flow  $Q$ , extraction,  $E$ , and permeability-surface area product,  $PS$ , is used for studies on the vascular bed of a whole organ. The difficulties are even more conspicuous when low flow states are to be studied and compared with normal flow states. Some factors concerning these problems will therefore be discussed in more detail.

##### 1 Blood flow $Q$

Throughout the present studies, the blood flow through the musculature of the dog's hind leg was estimated by measuring the total venous outflow in the iliac vein. In the control state, there are no obvious reasons to doubt that the total inflow of blood should be well represented by this measurement. This was confirmed in the present study when arterial inflow and venous outflow were simultaneously measured during the control state (see Chapter 9).

In previous studies with a surgical preparation similar to the present, venous outflow measurements were found to agree well with the arterial inflow measured with an electromagnetic flowmeter particularly during the control state (Sandegård 1974). During trauma, however, a significant increase of blood flow was noticeable for a shorter period of time (15 minutes) when measured from venous outflow than it was when measured from arterial inflow (30 minutes). This may indicate that the inflow measurements become inaccurate or that venous outflow measurements did not persistently follow the total blood flow through the vascular bed. The second explanation would be the development of a collateral venous outflow leading to a smaller increment in venous outflow as compared to arterial inflow for an initial period after trauma.

In the present study the presence of recruitable outflow vessels in the leg musculature was clearly demonstrated when the iliac vein was compressed. The total flow in the leg was only minimally unchanged in relation to arterial inflow when the venous outflow was occluded by mass ligatures.

In conditions with a decreased perfusion pressure and a decreased arterial inflow, such as arterial hypotension and hemorrhagic hypotension, there may be a similar diversion of venous outflow affecting the measurement of blood flow.

However in a hyperaemic state such as normovolemic hemodilution the presence of recruitable collaterals may possibly interfere with the measurement of total blood flow from venous return. The correction for an underestimation of flow after normovolemic hemodilution would give an even higher flow with an unchanged or increased PS. Such a correction would, if anything, enforce the evidence leading to the conclusion of an increased PS and increased flow after normovolemic hemodilution.

## 2. Extractions E

a) I-antipyrine a lipid-soluble molecule. The transport through the capillary wall of lipid-soluble molecules is not only confined to the differently sized pores that allow water and lipid-insoluble (water-soluble) molecules to cross the endothelial cell layer. Data from studies by Renkin (1952, 1953) indicated that lipid soluble molecules also diffuse rapidly through the cell membrane material. The entire surface area of the capillary wall may thus be utilized for blood tissue exchange of a molecule like antipyrine which is lipid-soluble. The extraction of the molecule will be independent of blood flow within a wide flow range and therefore its clearance ( $Q \times E$ ) will vary only with varying flow. The actual value of fractional extraction will be close to unity in a vasodilated tissue but lower in the resting state where non-uniformity of capillary blood flow distribution will act as a physiological shunt (Chinard et al. 1955 Renkin 1955 1959 Landis and Pappenheimer 1963).

In the present study the extraction of I-antipyrine was 0.79 (mean control value) and it remained independent of flow in the various activated states.

b) Cr-EDTA a lipid-insoluble (water-soluble) molecule. The curves that were obtained for the fractional extraction of Cr-EDTA showed in their initial parts, variable and usually high extraction values soon after injection of isotopes (see Fig. 4). High values were interpreted as being due to interlaminar diffusion, giving rise to different dispersion intravascularly for the participating diffusible (Cr-EDTA or iodide) and indiffusible (Fesiderophillin) tracer the so-called Taylor-effect (Taylor 1954). A plateau of constant extraction values, appearing in several samples around peak time for the curve of non-exchanging tracer was taken as evidence that the Taylor-effect had subsided and that back diffusion was minimal. Extraction values were estimated only when a series of extraction values were found to form a plateau.

Low initial values for extraction rising towards the plateau have previously been seen in non-vasodilated tissue (Rose and Gorensky 1976).

In several earlier studies, the weighted means of the separate extraction have been calculated in order to lessen the effects of extraction variation with time (Crone 1961). Other methods have been proposed by Martín de Julián and Yudilevich (1964) and by Bassingthwaite et al (1970)

### 3 PS PS/Q quotient of Cr-EDTA or Iodide and back diffusion

In resting muscle one fourth to one half of the total capillary surface is perfused (Papenhelm 1953 Kjellmer 1965 Renkin et al. 1966 Mellander and Johansson 1968). It is often assumed that the blood flow is uniformly distributed in the open capillaries with respect to their individual surface, i.e. the capillaries have identical S/Q ratios, permeability and extraction values. This would give PS values calculated in accordance with the equation  $PS = -Q \ln(1 - E)$ .

Total clearance of a substance may be predominantly achieved by extraction in relatively well-perfused capillaries which would, therefore, only represent a part of the capillary population. It should be kept in mind, however, that the clearance in capillaries receiving a larger fraction of blood cannot compensate for the decreased clearance in less effective capillaries (Renkin 1959 Renkin and Rosell 1962, Renkin 1961 1964 Renkin et al. 1966). If a number of capillaries were to be obstructed, PS cannot remain unchanged unless the degree of non-uniformity among remaining perfused capillaries is decreased or more capillaries recruited.

Non-uniformity of S/Q ratios and, hence, capillary blood flow distribution leads to a lower value of calculated PS product during control and may, if enhanced during activated states, explain additional apparent reductions of PS.

Changes of calculated PS are known to be induced by changes of blood flow. This is the case both in resting and vasodilated skeletal muscle (e.g. Renkin 1959 Trap-Jensen and Lassen 1970 Appelgren 1972, Pasake 1977 Rippe et al. 1978). The change in PS with flow, particularly prominent at lower flow levels, represents a functional rather than a geometrical alteration of the capillary surface.

In studies on blood tissue exchange, vasodilatation is often induced in order to reduce the effects of non-uniformity of capillary blood flow. In the present kind of experiments, such a procedure would probably induce too deleterious effects on central hemodynamics in the animal and was for this reason not practised.

It is, however, evident that experimentally observed changes in PS have to be evaluated in relation to any existing changes in flow. In a low flow state, factors related to the duration and magnitude of prevailing perfusion pressure may also have a specific bearing on PS (Appelgren 1972 cf. Järhult and Mellander 1974). At very low flow levels, PS measurements may very well become erroneous as a consequence of back diffusion, but also due to high PS/Q quotients, indicating flow-limitation, this is to be expected when the value of PS/Q is exceeding 0.5 (Goreau et al. 1970, Bassingthwaite 1974 Guller et al. 1975 Rippe 1978). In the present study the value of the PS/Q quotient was usually well below this figure.

Even if the total blood flow through a musculature were to be accurately measured it is not necessarily representing the total blood flow through the open capillaries (Renkin 1959 Renkin et al. 1966 Michel 1971). The existence in the resting muscle of the open-

ing in activated states of short arterio-venous interconnections (shunts) would produce very low PS/Q quotients which weighted for their flow in the calculation of total PS/Q would result in low PS values for the entire capillary bed.

#### 4 Blood to tissue vs tissue to blood transport

In some series of the present experiments (hemorrhage and hemodilution) two different methods were employed to study the blood tissue exchange. An attempt has been made to present the data from these methods in a conformed manner in extraction(Cr-EDTA)/flow diagrams.

It has occasionally been argued that indicators delivered to the tissue via the arterial blood stream would always stand a better chance to reach well-functioning capillaries than indicators which are directly deposited in the interstitial fluid. However any change in the number or in the functional capacity of those capillaries (per unit tissue weight or volume) which participate in diffusion transport, would affect the over-all diffusion capacity of the capillary bed regardless of the direction of the diffusional transfer and it is only when the tissue produces a considerable additional extravascular diffusion barrier for substances like  $^{51}\text{Cr}$ -EDTA that PS(tissue to blood) will be less than PS(blood to tissue)

In the situations where data were obtained simultaneously from the indicator dilution technique and the local clearance method, these data were in agreement during hemorrhagic hypotension and diverse during normovolemic hemodilution. Possible explanations for this finding will be discussed below

#### B The control state

In the present investigation, blood tissue exchange was studied in a resting skeletal musculature. The mean blood flow was 4.6 ml/min/100 g, the mean PS for Cr-EDTA 1.15 ml/min/100 g. The PS values were not significantly related to the flow values, the two parameters should be imagined as highly independent of one another each being related to the vascular tone prevailing in a different serial segment of the blood vessels. The large scattering of Q and PS values may to a great extent, depend on the varying degree of influence exerted on the blood vessel musculature by the wide range of mechanisms that is normally operative to govern blood flow and blood tissue exchange (Mellander and Johansson 1968)

The distribution of control PS values for Cr-EDTA is not a Gaussian one. This has also been observed in other studies on diffusion solute exchange across the capillary wall (e.g. Crone et al. 1978, cf. Crone and Christensen 1979). The skewed curve of distribution (see Fig. 6) is reminiscent of the one observed for values of the filtration coefficient (e.g. Pappenheimer et al. 1951 Michel et al. 1974) and indicative of a variation in the permea-

bility properties between individual capillaries or capillary beds. The possible causes for the variation in the values of PS (and filtration coefficient) are not, as yet, fully understood.

No significant correlations were found to exist between the values for Q and PS and various background variables which could possibly be related to the degree of circulatory disturbance at the time for control measurements.

Although much of the spread in magnitude of PS (and flow) changes is due to control differences, these differences, however, are not necessarily produced by a varying severity of preparation trauma (see Chapter 4). Furthermore the mode of reaction in an activated state is often more important physiologically from a relative quantitative or qualitative than from an absolute quantitative point of view.

The control Q and PS values are of some interest in relation to changes induced in different activated states since the magnitude of the changes in paired comparisons could be expected to depend on the initial level of the control value (varying from preparation to preparation). This was clearly demonstrated by Renkin and Rosell (1967), at sympathetic chain stimulation the magnitude of the change in resistance was seen to depend on the chosen rate of blood flow and on the vascular resistance which was prevailing in the control state. The change of PS was not influenced by variation of blood flow nor was it dependent on the simultaneously induced change in resistance. Apart from concluding that the change in PS was not effected by the increase in vascular resistance and that the resistance change was related to the level of vascular tone in control the authors also observed that the magnitude of the individual change in PS depended heavily on the difference of PS in control.

## C. The activated state

### 1 The activated state with a decreased blood flow

a. Regional arterial hypotension. In previous studies on the effects of regional arterial hypotension on the tissue-to-blood transport of low molecular size solutes, it was found that PS was more markedly and more consistently reduced (more than 50 %) following a one hour period of hypotension than within the first hour (Appelgren 1972). During the first hour there was an unsteady extraction rate as demonstrated by uneven slopes in the elimination rate curves (local clearance method). Several other studies on hypotension, including hemorrhagic shock, have also indicated that the time factor is of importance. Signs of autoregulation of the capillary pressure due to changes of the precapillary myogenic activity eventually disappear during prolonged arterial hypotension (Lewis and Mellander 1964, Jarhult and Mellander 1974) and fluid filtration from blood to tissue is seen to take place as a consequence of an increased post-capillary resistance (Mellander and Lewis 1963, Jarhult and Mellander 1974).

Järhult and Mellander (1974) also noted that the level to which perfusion pressure was reduced was decisive as to whether an increase of post-capillary resistance should arise during short term hypotension. The critical pressure level appeared to be 40 mm Hg. Such an increase of post-capillary resistance is believed to be due to rheological factors, also causing an obstructed and non-uniform for diffusion transport less effective, capillary blood flow by venular stasis.

Thus, the extent of blood tissue exchange during hypotension may be expected to depend to some extent, on the level of arterial pressure during short term hypotension and on the duration of the hypotension period during prolonged hypotension.

The flow disturbance caused by capillary obstruction should markedly affect PS which is known to be highly sensitive to changes in the number of perfused capillaries. This has been observed both in studies with the indicator diffusion method and with the local clearance method (Appelgren 1972, Rippe et al. 1978). The capillary coefficient for filtration on the other hand, appears to increase during regional arterial hypotension (Cobbold et al. 1963, Järhult and Mellander 1974) unless the hypotension is accompanied by massive capillary obstruction (Rippe et al. 1978).

Partly in order to estimate the flow-dependence of PS a series of experiments was made where the arterial perfusion pressure was regionally reduced. As a result of the arterial hypotension PS for Cr-EDTA was seen to decrease. On the basis of the experiments, however, it cannot be decided to what extent the PS reduction is a consequence of the flow-dependence of PS per se or if any part of the reduction does in fact correlate with an absolute decrease in capillary surface area due to capillary obstruction by rheological factors, such as retardation of blood corpuscles.

■ **Hemorrhagic hypotension** Changes which are induced by acute hypovolemia in the blood circulation of skeletal muscle seem to depend, among other things, both on the time factor and on the magnitude of the changes in perfusion pressure. At higher perfusion pressures, often initially present during hemorrhagic hypotension, the precapillary resistance is relatively more increased than the post-capillary. As the hypotension period is prolonged, precapillary vessels tend to dilate under the influence of locally accumulated metabolites. The initial fluid absorption into the blood, enhanced by hyperosmolality will eventually decline and in very late stages of hemorrhagic shock both hydrostatic and probably also osmotic forces will initiate filtration (Mellander and Lewis 1963, Lundgren et al. 1964, Mellander and Öberg 1967, Järhult 1973, 1975).

Acute hemorrhage has been found to give rise to a decrease in capillary diffusion capacity apparently due to a reduction in the number of capillaries which participate in blood tissue exchange. Appelgren (1972) found that acute hemorrhagic hypotension, at perfusion pressures above 60 mm Hg, after one hour induced a 50 % reduction of PS. Within the first hour the transport of iodide from the tissue to the blood was found to be unstable at reduced blood flow. Very similar results were obtained when the blood flow to

the hind leg, at comparable perfusion pressure was reduced by occlusion of the aorta and the transport studied during a similar course of time. These findings seem to be in line with observations indicating that rheological changes produced by hypotension require some time to develop and seem to be located towards the venous side in the larval bed (Jirhult and Melander 1974). Appelgren found an additional PS reduction appearing at perfusion pressures lower than 50 -- 60 mm Hg. He postulated that this additional PS reduction was caused by an increased heterogeneity of capillary blood flow with reduction of the perfusion pressure rather than any further reduction in the number of perfused capillaries.

Signs of a widespread capillary obstruction following acute hemorrhage were also found by Baeckström et al. (1971). CFC was reduced to 40 – 50 % of control value while flow resistance was occasionally increased six fold.

Discussing the relationship between capillary blood flow and coefficients for capillary filtration and diffusion, Renkin (1969) emphasized that diffusion transport is more sensitive than filtration to changes in capillary blood flow. To explain disproportionate changes of CFC and PS he argued that if flow distribution is controlled by a graded activity of terminal arterioles and precapillary sphincters, such a mechanism would change PS more than CFS. There are several studies to support this theory (e.g. Renkin et al. 1966).

When the peripheral resistance was increased six-fold by a precapillary obstruction induced by intravascular injection of microspheres into a vasodilated preparation, Rippe et al. (1978) found CFC to decrease to approx. 45 %, whereas ES decreased to less than one fourth of its control value. The PS decrease obtained is of the same order of magnitude as the maximal decrease seen to follow sympathetic adrenergic vasoconstriction (Renkin and Rosell 1962, Renkin 1967). However, sympathetic adrenergic vasoconstriction is known to subside soon after the onset of hemorrhagic hypotension (Mellander and Lewis 1963, Lundgren et al. 1964) and leads, *per se*, only to a smaller initial and short-lasting reduction of CFC (see Gebhold et al. 1963 cf. Celander and Folkow 1953).

If the effects of hemorrhage were to be similar to those produced by microsphere injection or sympathetic vasoconstriction, one would possibly expect, in comparison with CFC, a disproportionately large or in the latter case, only an early and transient reduction of PS. However in the present investigation, a more moderate and sustained decrease of PS was found, the magnitude of which (50%) is essentially the same as that reported earlier in similar studies (Appelgren 1972, PS, Backstrom et al. 1971 CFC). One possible explanation may be that there is, in acute hypovolemia, an almost total cessation of exchange processes in a certain number of capillaries, filtration and diffusion would then be more similarly reduced and both in proportion to the number of obstructed capillaries. Such a condition would mimic, at least during initial stages, an all-or-none action of individual capillaries rather than a graded activity of vasomotion (cf Renkin 1969).



In the present study acute hemorrhagic hypotension was seen to lead to a decrease in PS for Cr-EDTA. The first measurements during the hypotension period were carried out when at least one hour had elapsed since the onset of bleeding. Consequently the data do not permit any conclusions concerning the exchange capacity during the earlier stages of the hypotension period.

In comparison with the results obtained from a small number of experiments on regional arterial hypotension the reduction in PS of  $^{51}\text{Cr}$ -EDTA by the indicator dilution method was less equivocal and statistically significant when first measured between one and two hours after the onset of bleeding. The magnitude of the PS reduction was the same as when measured by the local clearance method.

## 2. The activated state with a variable blood flow

**Injury** Ten experiments were carried out to study the changes of blood flow and PS for Cr-EDTA which were produced by a prolonged and widespread traumatization of the musculature of the dog's hind leg. In five experiments, the blood flow was found to increase by 100 – 168 per cent when measured within the first few minutes after the injury the flow subsequently returned to control level during the following hour. In the other experiments, the blood flow decreased by 40 – 88 per cent and remained somewhat low in comparison with control. In most experiments (8/9 one excluded), PS was found to decrease. This is in contrast to normal skeletal muscle where a coordinated, adaptive response to increased metabolism is seen to take place characterized by a simultaneous increase in blood flow and in capillary filtration and diffusion capacities (Lewis and Melander 1962 Cobbold et al 1963 Kjellmer 1965 Renkin et al 1966). At metabolic vasodilatation produced by intermittent muscular contraction the increase in flow leads to an increase in PS. This was observed by Renkin et al. (1966). They also found that the return of blood flow towards control level, after cessation of the muscular work, was less rapid if perfusion rate was low in relation to the metabolic load.

Data from several earlier studies dealing with trauma indicate that injury is likely to induce a local reaction which includes vasodilatation and disturbance of capillary blood flow (e.g. Landis and Pappenheimer 1963). Metabolic factors are known to influence the vasomotion of tissues and are capable to dilate the blood vessels, particularly the segments which are situated proximal to the capillaries. The metabolites thus counteract the contraction of precapillary resistance vessels and sphincters which may be produced by various mechanisms e.g. sympathetic adrenergic activity. If metabolic events resembling those occurring e.g. during and after muscular work would appear in connection with injury the vascular response to injury might be expected to display some similarities with the one produced by metabolic vasodilatation under more normal circumstances, such as muscular work.

In the present series of experiments it was not possible to measure changes of blood flow and PS during the period when the injury was produced. It cannot be excluded that the blood flow response during this stage is different from what is indicated by the measurements made during the first 10 minutes. The rate of production of vasodilating metabolites is a regular feature of the period after the trauma. If the production of these metabolites continues during the period the injury is produced there are reasons to expect an increased flow at least temporarily during this period.

Lin (1968) and Lewis and Lin (1970) found that the relative blood flow increase after trauma was markedly reduced or absent in animals subjected to sympathectomy. This could indicate that the response to trauma may depend on the antagonistic nature of sympathetic adrenergic nerve activity and vasoactive substances, released by injury (cf Green 1961).

In the present study carried out on a large innervated vascular bed, an increase of total blood flow was not found to be a constant response to injury. If present it was of short duration and rarely as large as one expected from a total inhibition of vasoconstrictor nerve activity or from a moderate metabolic vasodilatation. The mean value for the increase in total blood flow was 36 per cent in the immediate post-traumatic period. In similar experiments, Lewis and Lin (1970) found, at an average, an initial 50 per cent increase in total blood flow, also measured from the total venous return in the iliac vein. Sandegård (1974) found a short-lasting, immediate blood flow increase of more than 100 per cent in the external iliac artery following the same kind of injury. For technical reasons, the blood flow in the iliac vein could not be measured simultaneously in the first few minutes following injury. The comparatively small increase in blood flow may indicate that the blood flow is rapidly regulated or, to some degree, underestimated.

Studying the blood tissue exchange during metabolic vasodilatation, Rankin et al (1966) found a sustained increase of PS during vasodilatation only when the blood flow was kept high and adequate for the metabolic load. If the blood flow was kept at a lower rate, PS all the same reached a maximum after half a minute during the period of muscular contraction (90 - 120 seconds) but it immediately started to decline towards control level while resistance to flow remained markedly decreased. After prolonged stimulation at low flow the PS response to muscular contraction could disappear entirely while the resistance response appeared to be unaffected. The authors postulated that, at very high levels of metabolism capillaries with very low  $s/q$  ratios may open up for an excessive flow leading to a PS fall due to a deteriorating, i.e. a non-adaptive distribution of capillary blood flow.

The present data reveal that PS may decrease during vasodilatation produced by injury. This finding could therefore indicate that the blood perfusion is, in the injured tissue, pathologically distributed and, possibly, inadequate in relation to the metabolic demand.

The metabolic demand of the injured muscle tissue may be considerably increased despite the variable response in blood flow and the predominant decrease in PS: the changes in these three parameters do not necessarily run in parallel in a pathophysiological situation. Thus, when blood flow is adequate in relation to the metabolic load precapillary sphincters are seen to respond in an adaptive manner in order to distribute the capillary blood flow uniformly. At inadequate flows, however, there may develop an inability for the sphincters to distribute the blood evenly among the capillaries. This could explain a rise in PS being smaller or more short-lasting than expected at a certain degree of vasodilatation, even if the sphincters initially may seem to be more sensitive to metabolite action than precapillary resistance vessels (cf. Mellander and Lewis 1963).

The decrease in PS should also be considered in relation to rheological factors. Following the kind of standardized injury which has been presently employed, local accumulation of erythrocytes eventually appears intravascularly and may be able to impair the capillary blood flow after approximately one hour at least at the post-capillary level (Knisely et al. 1947 Bigelow et al. 1949 Gelin 1956).

Other kinds of corpuscular obstruction (platelet aggregates, leucocytes) may possibly appear much earlier. The ability of the leucocyte to move along a capillary seems to be much reduced in hemorrhagic shock and may be directly related to the level of prevailing perfusion pressure (Bagge et al. 1979). It is not, as yet, known whether a similar capillary obstruction may occur in the capillaries of a musculature which has been subjected to severe contusion. The decrease in PS which is seen to occur early after injury cannot therefore at present, be accounted for by any known capillary obstruction by blood corpuscles, although such a mechanism deserves further investigation.

### 3 The activated state with an increased blood flow

**Acute normovolemic hemodilution.** The rapid single-injection multiple-indicator method was employed in fifteen experiments. In six experiments a local clearance method was simultaneously used to study the transport of the same water-soluble indicator (Cr-EDTA) in the opposite direction across the capillary wall.

An increasing amount of whole blood was replaced by dextran 70. The change in plasma flow at two levels of hemodilution (hematocrit 25–30 and 10–12%) was estimated by measurements of the total venous return and, for the local clearance method, by measurements of the Xenon clearance. Similar flow data were obtained from both methods, indicating an increase in plasma flow for each step of progressive hemodilution (55 and 107%). The increase of total blood flow which is seen to follow hemodilution is expected as a consequence of the decrease in whole blood viscosity. In a previous study total blood flow was found to be twice as large after extreme normovolemic hemodilution (Hct 10%) as in the control state: whole blood viscosity and total peripheral resistance were at the same time reduced to half their control values (Messmer et al. 1972). The

capillary blood flow estimated by local  $^{133}\text{Xe}$  clearance was three fold compared to control. However, it appeared as if remained unchanged in hemodilution; the increment in PS by any change in PS for  $^{51}\text{Cr-EDTA}$ , which was small by the local clearance method.

and to increase three-fold. This increase in perfusion capacity was not accompanied by a decrease in PS measured by the local

In the present study the total flow of plasma increased two fold after hemodilution to a hematocrit of 10–12% indicating a smaller change in total blood flow than was observed in the study previously mentioned. Some of the difference may depend on the blood flow response to the surgical preparation which was more extensive in the present study. Control flow and PS were both lower in the present investigation.

PS for  $\text{Cr-EDTA}$ , when estimated by the indicator dilution method, increased for each reduction of the hematocrit level; the values (based on plasma flow rates) were 65 and 122%. When the change in PS for  $\text{Cr-EDTA}$  was estimated by the local clearance method, much smaller changes were registered. At the first level of hemodilution there was an increase, at the second level a decrease in PS (13 and 12%) both values, however, were statistically insignificant.

It is interesting to note that the indicator dilution method and the local clearance method appear to be unanimous when recording the changes in blood flow and in PS during hemorrhagic shock whereas diverging results emerge for PS during hemodilution. The control states do not reveal any dissimilarities in these two series of experiments (cf. Tables IV and VII and Figs. 10 and 15; mean hematocrit = 45%). The two groups of data obtained by the local clearance method during hemorrhagic hypotension (decreased Q and decreased PS) and normovolemic hemodilution (increased Q and unchanged PS) are both in concord with previous findings (Appelgren 1977; Messmer et al. 1972); comparable data are not known to exist for the indicator dilution method.

The response in PS measured by the indicator dilution method appears to be uniform. The control measurements indicate, in comparison, low levels of plasma flow within a flow range from which the changes in flow per se are expected to be highly influential on PS (e.g. Appelgren 1972b; Rappe et al. 1978).

The response in PS, measured by the local clearance method, may be explained by the fact that control measurements of plasma flow extend into the flow range where changes in flow elicit comparatively moderate changes in PS. For each measurement an intramuscular injection of isotopes with a separate location was made which may also contribute to obscuring the results by increasing the spread of values in the control state. This reason is supported by the wider confidence limit obtained for the change in plasma flow by the local clearance method (hemodilution, level 1: 101–239% = 138%; level 2: 126–311% = 185%) in comparison with the corresponding confidence interval for the change in plasma flow obtained by measurement of venous return (level 1: 117–184% = 57%; level 2: 167–276% = 109%). Cf. Figs. 14 and 15.

#### 4 Increased venous pressure

Capillary filtration capacity (CFC) and the capillary permeability surface area product (PS) are commonly found to be reduced when the transmural pressure is elevated (Mellander et al. 1964, Johnson 1964). This reaction is generally accepted to be due to a myogenic response to elevated smooth muscle tension with a closure of precapillary sphincters. A similar "teleologic" mechanism, by reduction of CFC has been suggested to explain the protection against oedema formation in the lower limb in man in the prone position (Mellander et al. 1964).

The blood-to-tissue transport at increased venous pressure has recently been studied by Åberg (1973) who found the extraction ratio raffinose/sucrose to approach unity in experiments on the cat hind leg. Åberg explained this by an acceleration of transport caused by convection during the filtration process.

However Fleming and Diana (1976, 1977) reported only a minimal effect of venous pressure elevation on blood-to-tissue transport of small molecules. They used a single-injection multiple-indicator dilution and isogravimetric technique in dog hindlimbs vasodilated with papaverine.

In the present study extraction of  $^{51}\text{Cr}$ -EDTA, did not appear to be affected by elevation of venous pressure more than expected from a small flow reduction at constant PS. This was the case whether venous pressure was temporarily elevated or continuously elevated for several hours. The findings are in keeping with the hypothesis that net transcapillary fluid movement does not to any considerable degree affect the transport of small molecules through the capillary from blood to tissue. A reduction of the capillary bed (S-reduction) at increased venous pressure – such as described by the authors mentioned above – but compensated for by a facilitation of blood to tissue diffusion at net filtration of fluid cannot be excluded with the present experimental design. However the present results are in agreement with the observations by Fleming and Diana (1976, 1977).

In contrast, Lundgren and Mellander (1967) reported an increased transport from tissue to blood during net transcapillary fluid movement regardless its direction. In this state concentration gradients in the tissue may be of greater importance than in transport from blood to-tissue (Crone and Garlick 1970, Dahlberg and Lewis 1976). Such concentration gradients may be more or less dispersed by an increased paracapillary flow.

The extractions of the lipid-soluble  $^{125}\text{I}$ -antipyrine seemed to be maximal under control flow conditions and were not affected by short or prolonged increase of venous pressure.

## SUMMARY AND CONCLUSIONS

The blood tissue exchange of hydrophilic small solutes (Cr-EDTA, Iodid soluble substance (Iodoantipyrine) has been studied in the skeletal muscle

The single-injection multiple-indicator method and to some extent the indicator dilution method were employed

The vascular bed in the skeletal muscle was not initially controlled by any vasodilating procedure. A great deal of the differences that were demonstrated between the capillary beds of individual preparations may be due to this fact which also constitutes a limitation with regard to the interpretation of the results.

The main parameters were blood flow  $Q$  and (for lipid-insoluble indicators) extraction of indicators,  $E$ , and capillary diffusion capacity  $PS$ . The interrelationships of these parameters in the control state and their response to changes induced by regional arterial hypotension, hemorrhagic hypotension injury normovolemic hemodilution and graded obstruction of the iliac vein were studied. The evaluation of the results was greatly facilitated by a computerized statistical analysis of the entire group of control data and of the data obtained in each series of experiments.

The extraction of I-antipyrine, approx. 0.80 was independent of changes in the blood flow rate and in any of the activated states.

At decreased blood flow  $PS$  for Cr-EDTA decreased. The extent to which rheological factors, such as capillary obstruction by corpuscular blood elements, may have contributed to cause this decrease, could not be evaluated.

After a period of one to two hours of hemorrhagic hypotension at an arterial blood pressure of approximately 50 mm Hg, a 50 % reduction of  $PS$  for Cr-EDTA was estimated. The decrease in  $PS$  was the same and statistically significant, for both the indicator dilution method and the local clearance method. The results are in accordance with previous studies on capillary blood flow following acute hemorrhage.

As a result of injury  $PS$  for Cr-EDTA usually decreased markedly and tended to rise during the following hour. The change in  $PS$  measured by the indicator dilution method, seemed to be independent of the response in blood flow and was present from the very beginning of the observation period even in those animals which initially displayed a considerable increase in blood flow. The results indicate that the local distribution of capillary blood flow may severely and rapidly deteriorate when skeletal muscle is injured by powerful contusion and that the local blood flow in the anesthetized dog, may decrease or increase as a result of such an injury.

A successive increase in plasma flow was seen to characterize acute normovolemic hemodilution. Portions of the blood volume were replaced, step by step by equal amounts of dextran 70 a colloid plasma substitute. The increase in plasma flow exceeded 50 % at a hematocrit level of 25 - 30 % and at a level of 10 - 12 % the increase from control was

twice as large. The values for the changes in flow were nearly identical for the indicator dilution method (total venous return) and the local clearance method (Xenon clearance). Data obtained by the former technique indicated an increase in PS for Cr-EDTA. In contrast, no increase was registered when PS was measured by the local clearance method, used in six of the fourteen experiments. The reason for this discrepancy is not clear but may be sought for in the larger spread of values for changes in flow as registered by the tissue clearance method.

At graded compression of the iliac vein the venous return was partially diverted into collateral veins. The increase in venous pressure did not seem to affect PS for Cr-EDTA.

## ACKNOWLEDGMENTS

The research reported herein was supported in part by the United States Army through its European Research Office (6603) and by grants-in-aid from the Bayer AG, Federal Republic of Germany, the Göteborg Medical Society, Gothenburg, Sweden, the Swedish Association for Traffic- and Police-Injured, Stockholm, the Swedish Medical Research Council (B73-40P-7485-OA B 7 40X 74 07A, B75-40P-2485-OB, B7 40X-2042-09C, B77-7485 O A B 76 485 O-A1, Stockholm.

The author expresses his deepest appreciation to

Lennart Appelgren, Docent, Department of Anaesthesiology, University of Göteborg, for his unprecedented collaboration. Perseverance, clarity and order characterize the manner, in which he so eminently has provided guidance in the analysis and synthesis of data. His contribution is truly estimable.

Leif-Erik Celius, Professor, Department of Surgery I, University of Göteborg, for his personal concern and inspiring generosity. His dedicated leadership was a beacon and a constant source of inspiration throughout the studies. The support and advice rendered to the author are gratefully acknowledged.

Daved Lewis, Professor, Clinical Research Center, University of Linköping, for his guidance and teaching, particularly during the years his presence in Göteborg enlightened not only the author but so many other persons and problems, locally associated with research in surgery and microcirculation. His kindly shared much of his sound knowledge; the excellence of his criticism was always matched by the fairness.

Bo Eriksson, Fil. Lic., Department of Social Medicine, University of Göteborg for excellent assistance with the entire computerized statistical analysis of data.

Dr Charles Michel, University Laboratory of Physiology, Oxford, U.K., for his crucial comments on an early version of the manuscript.

The author is grateful to Dr Bengt Rippe, Department of Physiology, University of Göteborg, for helpful discussions.

Excellent technical assistance in the Laboratory of Rheology, Department of Surgery I was provided by Miss Lillemor Karlsson, Miss Ragnar Söderberg, Miss Elisebeth Sandström and Miss Monica Wallén.

Web-type acousticalization equipment was put at the author's disposal by the Department of Chemical Physiology and the Department of Clinical Chemistry. For all assistance and personal concern the author is greatly indebted to Mattias Anrell, Docent, Section of Nephrology, Department of Medicine I, Sven Lindstedt, Professor and Ragnar Nordberg, Docent, Department of Chemical Chemistry, University of Göteborg.

Miss Inger Isberg and Miss Karin Linnér take much credit for typing the manuscript. Mrs Barbro Johansson re-typed the entire manuscript before printing. Finally, Mr Ernez Parnitz is remembered with gratitude for his photographic work and Mr Holger Hansson for his devoted care of the animals.

The memory of Jack Adams-Ray is much cherished by many surgeons in Sweden. The author had the privilege as medical student, to be counselled and inspired by this great teacher for whom surgery and physiology were always closely linked together.





## REFERENCES

(Some references are to be found in subsequent lists of publications under separate head-lines).

- Aberg, B. (1973). Restricted convection-diffusion across capillary walls. Doctoral thesis. Acta Univ Upsalienens 160 1-36.
- Andersen, A.M. and Ladefoged, J. (1967). Partition coefficient of 133-Xenon between various tissues and blood in vivo. Scand. J. Clin. Lab. Invest. 19 72-78.
- Appelgren, K.L. and Lewis, D.H. (1968). Capillary permeability-surface area product (PS) of Renkin in human skeletal muscle. Acta Med. Scand. 184 281-282.
- Appelgren, K.L. (1972). Perfusion and diffusion in shock. A study of disturbed tissue-blood exchange in low flow states in canine skeletal muscle by a local clearance technique. Doctoral thesis. Acta Physiol. Scand., Suppl. 378, 1-72.
- Appelgren, K.L. (1972a). Capillary flow and capillary transport in dog skeletal muscle at prolonged low perfusion pressure. Eur. Surg. Res. 4 221-233.
- Appelgren, K.L. (1972b). Effect of perfusion pressure and hematocrit on capillary flow and transport in hyperemic dog skeletal muscle of the dog. Microvasc. Res. 4 231-246.
- Appelgren, K.L. and Lewis, D.H. (1973). Disturbances in capillary transport of skeletal muscles of the canine hind-leg by large preparative procedures in the abdomen. Bibl. Anat. 12, 381-385.
- Beckström, P., Folkow, B., Kowák, A.G.B., Lofving, B. and Öberg, B. (1971). Evidence of plugging of the microcirculation following acute hemorrhage. 6th Europ. Conf. Microcirc., Aalborg 1970. (J. Dzitel and D.H. Lewis, eds.). Karger Basel, 16-20.
- Beggs, U., Arundson, B. and Lauritzen, C. (1979). White blood cell deformability and plugging of skeletal muscle microcirculation in hemorrhagic shock. (In press).
- Bigelow, W.G., Heimbecker, R.O. and Harrison, R.C. (1949). Intravascular agglutination (staged blood), vascular stasis and sedimentation rate of blood in trauma. Arch. Surg. 59 667-690.
- Chen, S. (1967). Role of the sympathetic nervous system in hemorrhage. Physiol. Rev. 47 214-238.
- Chen, S. (1969). Blood rheology and its relation to flow resistance and transcapillary exchange with special reference to shock. Advanc. Microcirc. Karger Basel/New York, 2, 89-103.
- Chinard, F.P., Vosburgh, G.J. and Enns, Th. (1955). Transcapillary exchange of water and of other substances in certain organs of the dog. Am. J. Physiol. 183 221-234.
- Cobbold, A., Folkow, B., Kjellmer I. and Melander, S. (1963). Nervous and local chemical control of pre-capillary sphincters in skeletal muscle as measured by changes in filtration coefficient. Acta Physiol. Scand. 57 180-192.
- Crooks, C. (1963). Permeability of capillaries in various organs as determined by use of the indicator diffusion method. Acta Physiol. Scand. 58 292.
- Crooks, C. and Garlick, D. (1970). The penetration of insulin, sucrose, mannitol and tritiated water from the interstitial space in muscle into the vascular system. J. Physiol. 210, 387-404.
- Curry, F.E., Mason, J.C. and Michel, C.C. (1973). The effect of bovine  $\gamma$ -globulin and  $\text{Ca}^{2+}$  on the filtration coefficient of individual frog mesenteric capillaries. J. Physiol. (London) 234 43P.
- Dahlberg, J.B. and Lewis, D.H. (1973). Effect of hemorrhagic shock on capillary transport in dog skeletal muscle. Bibl. Anat. 12, 297-302.
- Dahlberg, J.B. and Lewis, D.H. (1975). Capillary flow and transport during hemodilution. Bibl. Haemat. 41 34-43.
- Dahlberg, J.B. and Lewis, D.H. (1976). Bidirectional capillary transport of small molecules. In Microcirculation 2 (J. Geryson and W. Zang, eds.) Plenum Press, New York and London, 125-131.
- Dawson, L., Appelgren, K., Gelan, L.-F. and Haglund, E. (1979). Skeletal muscle microcirculation and oxygenation in experimental intestinal shock. (In press).
- Dawson, L., Gelan, L.-F. and Haglund, E. (1979b). Hemodilution and oxygen transport to tissue in shock. (In press).

- Dawidson, I., Eriksson, B., Gelin, L.-E. and Soderberg, Rigmor (1979c). Oxygen consumption and recovery from surgical shock in rats. A study on the efficiency of different plasma substitutes. (In press).
- Fleming, B.P. and Diana, J.N. (1976). Effects of transcapillary fluid movement on solute exchange in isolated dog hindlimb. *Microvasc. Res.* 11: 118.
- Fleming, B.P. and Diana, J.N. (1977). Interaction between fluid movement and solute exchange in the isolated dog hindlimb. *Bibl. Anat.* 15: 483-488.
- Gelin, L.-E. (1956). Studies in anemia of injury. Doctoral thesis. *Acta Chir. Scand.*, Suppl. 210: 1-130.
- Gelin, L.-E. (1962a). Fluid substitution in shock. In *Shock, Pathogenesis and Therapy*. An international symposium. (K.D. Bock, ed.) Springer Verlag OHG: 332-347.
- Gelin, L.-E. (1962b). Rheologic disturbances and the use of low viscosity dextran in surgery. *Rev. Surg.* 19: 385-400.
- Gelin, L.-E. (1973). Intravascular aggregation of blood cells and tissue metabolic defects. *Adv. Exp. Med. Biol.* 37: 647-656.
- Gosselin, R.E. (1967). Blood-tissue exchanges of radioactive solutes in cardiac and skeletal muscle: Influences of isoproterenol, methacholine and nitroglycerin. *Fed. Proc.* 26: 398.
- Green, H.D. (1961). Physiology of peripheral circulation in shock. *Fed. Proc.* 20, Suppl. 9: 61.
- Galler, B., Yipintsoi, T., Orris, A.L. and Basingthwaigite, J.B. (1975). Myocardial sodium extraction at varied coronary flows in the dog. *Circ. Res.* 37: 359-378.
- Hamilton, W.F., Moore, J.W., Kinsman, J.M. and Spurling, R.G. (1932). Studies on the circulation. IV. Further analysis of the injection method, and of changes in hemodynamics under physiological and pathological conditions. *Am. J. Physiol.* 99: 534.
- Hymen, C., Rapaport, I., Saul, A.M. and Miron, M.E. (1952). Independence of capillary filtration and tissue clearance. *Am. J. Physiol.* 168: 674-679.
- Järbult, J., Lundvall, J., Mellander S. and Tibblin, S. (1972). Osmolar control of plasma volume during hemorrhagic hypotension. *Acta Physiol. Scand.* 85: 142-144.
- Järbult, J. (1973). Osmotic fluid flow from tissue to blood during hemorrhagic hypotension. *Acta Physiol. Scand.* 89: 213-226.
- Järbult, J. and Mellander S. (1974). Autoregulation of capillary hydrostatic pressure in skeletal muscle during regional arterial hypo- and hypertension. *Acta Physiol. Scand.* 91: 32-41.
- Järbult, J. (1975). Osmolar control of the circulation in hemorrhagic hypotension. An experimental study in the cat. *Acta Physiol. Scand.*, Suppl. 423.
- Kety S.S. (1949). Measurement of regional circulation by the local clearance of radioactive sodium. *Am. Heart J.* 38: 321-328.
- Kety S.S. (1957). Determinants of tissue oxygen. *Fed. Proc.* 16: 666-670.
- Kinsman, J.M., Moore, J.W. and Hamilton, W.F. (1929). Studies on the circulation. I. Injection method: physical and mathematical considerations. *Am. J. Physiol.* 89: 322.
- Kjellmer I. (1965). Studies on exercise hyperaemia. *Acta Physiol. Scand.* 64, Suppl. 244: 1-27.
- Kinsky M.H., Bloch, E.H., Eliot, T.S. and Warner L. (1947). Sludged blood. *Science* 106, 431-440.
- Lands, E.M. (1927). Micro-injection studies of capillary permeability. II. Relationship between capillary pressure and the rate at which fluid passes through the walls of single capillaries. *Am. J. Physiol.* 81: 17-38.
- Larven, N.A. and Trap-Jensen, J. (1970). The validity of the indicator dilution method for measuring the capillary diffusion capacity for  $^{51}\text{Cr}$ -EDTA in hyperaemic skeletal muscle. *Eur. J. Clin. Invest.* 1: 118-123.
- Levick, J.R. and Michel C.C. (1970). The effect of bovine albumin on the permeability of frog mesenteric capillaries. *J. Physiol.* 210: 36-37 P.
- Lewis, D.H. and Mellander S. (1962). Competitive effect of sympathetic control and tissue metabolites on resistance and capacitance vessels and capillary filtration in skeletal muscle. *Acta Physiol. Scand.* 56: 162-188.

- Lewis, D.H. and Lin, R.C. Jr (1970). Studies on the circulatory pathophysiology of trauma. I. Effect of acute soft tissue injury on nutritional and non-nutritional shunt flow through the hind-leg of the dog. *Acta Orthop Scand.* 41 17
- Lin, R.C., Koztrowska, E., Bergantz, S.-E. and Gelle, L.-E. (1968). Blood flow property changes following infusion with Dextran 40 and Dextran 70. *Rev. Surg.* 25 151
- Linde, B., Chikholm, G. and Rosell, S. (1974). The influence of sympathetic activity and histamine on the blood-tissue exchange of solutes in canine adipose tissue. *Acta Physiol. Scand.* 92, 145-155
- Lin, C. T. (1968). Circulatory response to muscle trauma following denervation and hemorrhage in dogs. *J. Trauma* 8 75
- Lundgren, O., Lundvall, J. and Mellander, S. (1964). Range of sympathetic discharge and reflex vascular adjustments in skeletal muscle during hemorrhagic hypotension. *Acta Physiol. Scand.* 62, 380-390
- Lundgren, O. and Mellander, S. (1967). Augmentation of tissue-blood transfer of solutes by transcapillary filtration and absorption. *Acta Physiol. Scand.* 70 26-41.
- Mason, J.C., Michel, C.C. and Took, J.E. (1973). The effect of plasma proteins and capillary pressure upon the filtration coefficient of frog mesenteric capillaries. *J. Physiol. (London)* 229 15P
- Mason, J.C., Curry F.E. and Michel, C.C. (1977). The effects of proteins upon the filtration coefficient of individually perfused frog mesenteric capillaries. *Microvasc. Res.* 13 185-202.
- M. Bender S. (1960) Comparative studies on the adrenergic neurohumoral control of resistance and capacitance blood vessels in the cat. *Acta Physiol. Scand.* 50, Suppl. 176 1-86.
- Mellander, S. and Lewis, D.H. (1963). Effects of hemorrhagic shock on the reactivity of resistance and capacitance vessels and on capillary filtration in cat skeletal muscle. *Circ. Res.* 13 105-118.
- Mellander, S., Öberg, B. and Odellström, H. (1964). Vascular adjustment to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer. *Acta Physiol. Scand.* 61 34-48.
- Mellander, S. and Öberg, B. (1967). Transcapillary fluid absorption and other vascular reactions in the human forearm during reduction of the circulating blood volume. *Acta Physiol. Scand.* 71, 37-46.
- Mellander, S. (1971) Interactions of local and nervous factors in vascular control. *Aspitol* 8, 187-201
- Messmer, K., Lewis, D.H., Sunder-Plassmann, L., Kjövelorn, W.P., Mendler, N. and Holper, K. (1972). Acute normovolemic hemodilution. Changes of central hemodynamics and microcirculatory flow in skeletal muscle. *Eur. Surg. Res.* 4 55-70.
- Michel, C.C. (1971). The calculation of capillary permeability from arteriovenous differences in uniform and non-uniform capillary bed. In *Research in Physiology* (liber amicorum in honour of Prof. Chandler McCuskey Brooks. (P. F. Kao, K. Kotzani and M. Vassallo eds.), Aulo Gaggi Publisher, Bologna, 1971 357
- Michel, C.C., Mason, J.C., Curry F.E., Took, J.E. (1974). A development of the Landis technique for measuring the filtration coefficient of individual capillaries in the frog mesentery. *Quart. J. Exp. Physiol.* 59 283-309
- Öberg, B. (1964). Effects of cardiovascular reflexes on net capillary fluid transfer. *Acta Physiol. Scand.* 62, Suppl. 229 1-98.
- Paasik, W.P. (1977). Capillary permeability in skeletal muscle. *Acta Physiol. Scand.* 101 1-14.
- Pappenheimer J.R. and Soto-Rivera, A. (1948). Effective osmotic pressure of the plasma proteins and their quantities associated with the capillary circulation in the hind limbs of cat and dogs. *Am. J. Physiol.* 152, 471-491
- Pappenheimer J.R., Reekun, E.M. and Borrero, L.M. (1951). Filtration, diffusion and molecular moving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *Am. J. Physiol.* 167 13-46.
- Partridge N.F., Swartz C.R., Shaw, D.H. and Fox, L.J. (1971). Perfusion heterogeneity in skeletal muscle using tritiated water. *Am. J. Physiol.* 220. 1107-1115

- Renkin, E.M. (1952). Capillary permeability to lipid-soluble molecules. *Am. J. Physiol.* 168: 538.
- Renkin, E.M. (1953). Capillary and cellular permeability to some compounds related to antipyrine. *Am. J. Physiol.* 173: 125-130.
- Renkin, E.M. (1955). Effects of blood flow on diffusion kinetics in isolated perfused hind-legs of cats. A double circulation hypothesis. *Am. J. Physiol.* 183: 125-136.
- Renkin, E.M. (1959). Transport of potassium-42 from blood to tissue in isolated mammalian skeletal muscles. *Am. J. Physiol.* 197: 1205-1210.
- Renkin, E.M. (1961). Mathematical analysis of the relation between uniformity of capillary circulation and efficiency of transcapillary exchange. Abstracts, International Biophysics Congress, Stockholm 1961: 135.
- Renkin, E.M. and Rosell, S. (1962). The influence of sympathetic adrenergic vasoconstrictor nerves on transport of diffusible solutes from blood to tissues in skeletal muscle. *Acta Physiol. Scand.* 54: 223-240.
- Renkin, E.M. (1964). Normal regulation of tissue circulation. In *Effects of Anesthetics on the Circulation*, (M.L. Price and P.J. Cohen, eds.) Springfield, Ill. Thomas: 171-181.
- Renkin, E.M., Hodicki, O. and Shoshan, R.M. (1966). Influence of metabolic vasodilatation on blood-tissue diffusion in skeletal muscle. *Am. J. Physiol.* 211: 87-98.
- Renkin, E.M. (1967). Blood flow and transcapillary exchange in skeletal and cardiac muscle. I. Coronary Circulation and Energetics of the Myocardium. (G. Marchetti and B. Taccardi, eds.) Basel, S. Karger: 18-30.
- Renkin, E.M., Joyner W.L., Sloop, C.H. and Watson, P.D. (1977a). Influence of venous pressure on plasma-lymph transport in the dog's paw: Convective and dispersive mechanisms. *Microvasc. Res.* 14: 191-204.
- Renkin, E.M., Watson, P.D., Sloop, C.H., Joyner W.L. and Curry E. (1977b). Transport pathways for fluid and large molecules in microvascular endothelium of the dog's paw. *Microvasc. Res.* 14: 205-214.
- Rippe B. (1978). Relationships between diffusion and convection across capillary walls. Experimental studies on continuous capillaries in cat and rat. Doctoral thesis. Department of Physiology Gothenburg University.
- Rippe B., Kamiya A. and Folkow B. (1978). Simultaneous measurements of capillary diffusion and filtration exchange alterations in the capillary permeability surface area product (PS) and during shift in filtration-absorption. *Acta Physiol. Scand.* 102: 52A-53A.
- Rippe B. and Stage L. (1978). An "on-line" colorimetric method for repeated rapid determination of capillary diffusion capacity. *Acta Physiol. Scand.* 102: 108-115.
- Rippe B., Kamiya, A. and Folkow B. (1979). Transcapillary passage of albumin, effects of tissue cooling and of increases in filtration and plasma colloid osmotic pressure. *Acta Physiol. Scand.* 105: 171-187.
- Sandegård, J. (1974). Vasodilatation in extremity trauma. Immediate hemodynamic changes in the dog hind leg. Doctoral thesis. *Acta Chir. Scand. Suppl.* 447: 1-32.
- Starling, E.H. (1896). On the absorption of fluid from the connective tissue spaces. *J. Physiol. (Lond)* 19: 312-36.
- Stewart, G.N. (1897). Researches on the circulation time and on the influences which affect it. IV. The output of the heart. *J. Physiol.* 22: 159-183.
- Stewart, G.N. (1921). The output of the heart in dogs. *Am. J. Physiol.* 57: 27.
- Stewart, G.N. (1921). The pulmonary circulation time, the quantity of blood in the lungs and the output of the heart. *Am. J. Physiol.* 58: 20.
- Strander, T. and Shepherd J.T. (1968). The effect in human of exercise on relationship between simultaneously measured  $^{133}\text{Xe}$  and  $^{24}\text{Na}$  clearances. *Scand. J. Clin. Lab. Invest.* 21: 99-107.

- Taylor G.I. (1954). The dispersion of soluble matter in solvent flowing slowly through tubes. *Proc. Roy. Soc. A* 219 186-203.
- Yudilevich, D.L., Renkin, E.M., Alvarez, O.A. and Bravo L. (1968). Fractional extraction and transcapillary exchange during continuous and instantaneous tracer administration. *Circ. Res.* 23, 325-336

## REVIEWS

- Aachheim, E. (1977). Passage of substances across the walls of blood vessels. Kinetics and mechanisms. In *Microcirculation*. (G. Kaley and B.M. Altura, eds.) University Park Press, Baltimore - London - Tokyo 213-249
- Chambers, R. and Zweifach, B.W. (1947). Inter cellular cement and capillary permeability. *Physiol. Revs.* 27 436-463
- Chisard, F.P. (1975). Estimation of extravascular lung water by indicator-dilution techniques. *Circ. Res.* 37 137-145
- Crone, C. (1970). Capillary permeability - techniques and problems. In *Capillary Permeability* (C. Crone and N.A. Lassen, eds.) Munksgaard, Copenhagen, 15-31.
- Crone, C. (1973). Capillary permeability II. Physiological considerations. In *The Inflammatory Process*. (B.W. Zweifach, L. Grant and R.T. McCloskey eds.) Academic Press, New York 2, 95-121
- Crone, C. (1974). Træk af kapillærfysiologiens udvikling. Festskrift, Københavns Univ. Bianco Lunos Bogtrykkeri A/S, Copenhagen.
- Crone, C. and Christensen, D. (1979). Transcapillary transport of small solutes and water. I. Cardiovascular Physiology III. (A.C. Guyton and D.B. Young, eds.) 18, 149-213
- Danielli, J.F. and Stock, A. (1944). The structure and permeability of blood capillaries. *Biol. Rev* 19 81-94.
- Florey H.W. (1964). The transport of materials across the capillary wall. *Quart. J. Exp. Physiol.* 49 117-128.
- Folkow B. (1955). Nervous control of the blood vessels. *Physiol. Rev* 35 629-663.
- Folkow B. (1956). The nervous control of the blood vessels. In *The Control of the Circulation of the Blood*. Suppl. (R.J.S. McDowell, ed.) Dawson, London, 1-83.
- Folkow B. (1960). Role of the nervous system in the control of vascular tone. *Circ.* 21, 760-764.
- Folkow B. and Møller S. (1970). Measurements of capillary filtration coefficient and its use in studies of the control of capillary exchange. In *Capillary Permeability* (C. Crone and N.A. Lassen, eds.) Munksgaard, Copenhagen, 614-627
- Guyton, A.C., Coleman, T.G. and Osminger H.J. (1972). Circulation. Overall regulation. *Ann. Rev. Physiol.* 34 13-46.
- Haddy F.J., Scott, J.B. and Grege, G.J. (1976). Peripheral circulation. Fluid transfer across the microvascular membranes. In *1st Review of Physiology Cardiovascular Physiology II*. (A.C. Guyton and A.W. Cowley eds.) 9 63-109
- Howe, C.R. (1974). Transport across the capillary wall. In *Water Transport in Cells and Tissues*. Monographs of the Physiological Society (K. Davson, A.D.M. Greenfield, W. Whittam and G.S. Brindley eds.) Edward Arnold, London, 362.
- Johnson, P.C. (1964). Review of previous studies and current theories of autoregulation. *Circ. Res.* 14 and 15 Suppl. 1 2-9
- Johnson, P.C. (1974). The microcirculation, and local and humoral control of the circulation. I. A.C. Guyton and C.E. Jones: MTP International review of science Cardiovascular physiology Butterworths, London. University Park Press, Baltimore, 163.
- Karnovsky M.J. (1968). The ultrastructure of bases of transcapillary changes. I. Biological Interfaces: Flows and exchanges. Little Brown & Co Boston, 64-95.
- Karnovsky M.J. (1968). The ultrastructural bases of transcapillary exchanges. *J. Gen. Physiol.* 52, 64-95
- Karnovsky M.J. (1970). Morphology of capillaries with special reference to muscle capillaries. In *Capillary Permeability* (C.A. Crone and N.A. Lassen, ed.) Munksgaard, Copenhagen, 341-350.

- Kety S.S. (1951). The theory and applications of the exchange of inert gas at the lungs and tissues. *Pharmacol. Rev.* 3 1-41
- Krogh, A. (1929). The anatomy and physiology of the capillaries. New Haven. Yale Univ. Press, 422
- Krogh, A. (1946). The active and passive exchanges of inorganic ions through the surfaces of living cells and through living membranes generally. *Proc. Roy. Soc. B.* 133 140-200.
- Landis, E.M. (1934). Capillary pressure and capillary permeability. *Physiol. Revs.* 14 404-481
- Landis, E.M. (1946). Capillary permeability and the factors affecting the composition of capillary filtrate. *Ann. N.Y. Acad. Sci.* 46, 713-727
- Landis, E.M. and Pappenheimer J.R. (1963). Exchange of substances through the capillary walls. In *Handbook of Physiology* vol. 2 sec. 2, Circulation. (W.F. Hamilton and P. Dow eds.) American Physiological Society Washington, D.C., 961-1034.
- Leonard, E.F. and Jorgensen, S.B. (1974). The analysis of convection and diffusion in capillary beds. *Ann. Rev. Biophys. Bioeng.* 3 293-339
- Left, J.H. (1965). The ultrastructural basis of capillary permeability. In *The Inflammatory Process* (B.W. Zweifach, L. Grant and R. McCluskey eds.) Academic Press, New York and London, 121-159
- Left, J.H. (1973). Capillary permeability I. Structural considerations. In *Inflammatory Process* (B.W. Zweifach, L. Grant and R.T. McCluskey eds.) Academic Press, New York, 2, 47-84
- Majno, G. (1965). Ultrastructure of the vascular membrane. In *Handbook of Physiology* vol. 2, sec. 3 Circulation. (W.F. Hamilton and P. Dow eds.) American Physiological Society Washington, D.C., 2293-2375
- Mayerson, H.S. (1963). Physiologic importance of lymph. In *Handbook of Physiology* vol. 2, sec. 2, Circulation. (W.F. Hamilton and P. Dow eds.) American Physiological Society Washington, D.C., 1035-1073
- McLander, S. and Johansson, B. (1968). Control of resistance, exchange, and capacitance functions in the peripheral circulation. *Pharmacol. Rev.* 20, 117-196
- McLander, S. (1970). Systemic circulation. Local control. *Ann. Rev. Physiol.* 32, 313-344.
- Michel, C.C. (1972). Flows across the capillary wall. In *Cardiovascular Fluid Dynamics* (D.J.L. Bergel, ed.) Academic Press, London, 2, 241-298
- Neil, E. (1962). Reflex mechanisms and the central nervous system. In *Shock: Pathogenesis and Therapy* (K.D. Bock ed.) Springer Verlag, 172-176.
- Onsager, L. (1931a). Reciprocal relations in irreversible processes. I. *Phys. Rev.* 37 405-426.
- Onsager, L. (1931b). Reciprocal relations in irreversible processes. II. *Phys. Rev.* 38 2265-2279
- Pappenheimer J.R. (1953). Passage of molecules through capillary walls. *Physiol. Rev.* 33 387-423.
- Renkin, E.M. and Pappenheimer J.R. (1957). Wasserdurchlässigkeit und Permeabilität der Capillärwände. *Ergeb. Physiol.* 49 59-126
- Renkin, E.M. (1964). Transport of large molecules across capillary walls. *Physiologist* 7 13-28
- Renkin, E.M. (1968). Transcapillary exchange in relation to the capillary circulation. *J. Gen. Physiol.* 52, 96s - 108s.
- Renkin, E.M. (1969). Exchange of substances through capillary walls. In *Circulatory and Respiratory Mass Transport*. A Ciba Foundation Symposium, 50-66.
- Renkin, E.M. (1977). Multiple pathways of capillary permeability. *Circ. Res.* 14 735-743
- Renkin, E.M. and Curry F.C. (1978). Transport of water and solutes across capillary endothelium. In *Handbook of Epithelial Transport*. (G. Giebisch, D.C. Tosteson and J.H. Ussing, eds.) Membrane Transport in Biology. Springer Verlag.
- Rowlett, R.D. and Harris, T.R. (1976). A comparative study of organ models and numerical techniques for the evaluation of capillary permeability from multiple-indicator data. *Math. Biosci.* 29 273-298.
- Stainby W.N. (1973). Local control of regional blood flow. *Ann. Rev. Physiol.* 35 151-168.
- Starverman, A.J. (1951). The theory of measurement of osmotic pressure. *Rec. Trav. Chim.* 70, 344-352.
- Ussing, H.H. (1953). Transport through biological membranes. *Ann. Rev. Physiol.* 15 1-20
- Webb, R.E. (1964). Rheology of blood in the microvasculature. *New Engl. J. Med.* 270 889-893
- Wilde W.S. (1955). Transport through biological membranes. *Ann. Rev. Physiol.* 17 17-36
- Zweifach, B.W. (1973). Microcirculation. *Ann. Rev. Physiol.* 35 117-150

## THEORY AND METHODS

- Chen, S (1971). A theory for the quantification of transcapillary exchange in the presence of shunt flow. *Circ. Res.* 29 173-180.
- Chisvard, F.P. Effros, R., Perl, W. and Silverman, M. (1967). Organ vascular and extravascular compartments in vivo. In *Compartments, Pools, and Spaces in Medical Physiology* (P.E. Bergner and C.C. Lushbaugh, eds.), AEC Symposium No. 11. Clearinghouse for Federal Scientific and Technical Information, National Bureau of Standards, U.S. Department of Commerce, Springfield, Virginia 22151 381.
- Croos, C. and Friedmen, J.J. (1976). A method for determining potassium permeability of single capillary. *Acta Physiol. Scand.* 96 13A.
- Dow P (1955). Dimensional relationships in dye-dilution curves from humans and dogs, with an empirical formula for certain troublesome curves. *J Appl. Physiol.* 7 399.
- Grim, E. (1953). Relation between pressure and concentration differences across membranes permeable to solute and solvent. *Proc. Soc. Exp. Biol. Med.* 83 195-200.
- Katchalsky A. and Kedem, O. (1962). Thermodynamics of flow processes in biological systems. *Biophys. J.* 2, 53-78.
- Kedem, O. and Katchalsky A. (1958). Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. *Biochim. Biophys. Acta* 27 229-246.
- Kedem, O. and Katchalsky A. (1961). A physical interpretation of the phenomenological coefficients of membrane permeability. *J. Gen. Physiol.* 45 143-179.
- Kedem, O. and Katchalsky A. (1963). Permeability of composite membranes. *Trans. Faraday Soc.* 59 1918.
- Lassen, N.A. and Trap-Jensen, J. (1968). Theoretical considerations on measurements of capillary diffusion capacity in skeletal muscle by the local clearance method. *Scand. J. Clin. Lab. Invest.* 21 108-115.
- Lassen, N.A. and Croos, C. (1970). The extraction fraction of capillary bed to hydrophilic molecules: Theoretical considerations regarding the single injection technique with discussion of the role of diffusion between luminal streams (Taylor effect). In *Capillary Permeability* (C. Croos and N.A. Lassen, eds.) Munksgaard, Copenhagen, 302-305.
- Lee, J.S. and Fronzek, A. (1970). An analysis on the exchange of indicators in single capillaries. *Microvasc. Res.* 2, 302-318.
- Lewitt, D.G. (1974). A new theory of transport for cell membranes pores. I. General theory and application to red cell. *Biochim. Biophys. Acta* 373 115.
- Lewitt, D.G. and Subramaniam, G. (1974). A new theory of transport for cell membranes pores. II. Exact results and computer simulation (molecular dynamics). *Biochim. Biophys. Acta* 373, 132.
- Martin de Johnin, P. and Yudilevich, D.L. (1964). A theory for the quantification of transcapillary exchange by tracer dilution curves. *Am. J. Physiol.* 207 162-168.
- Minor F and Zierler, K.L. (1954). On the theory of the indicator-dilution method for measurement of blood flow and volume. *J. Appl. Physiol.* 6, 731.
- Perl, W. (1962). Heat and matter distribution in body tissues and the determination of tissue blood flow by local clearance methods. *J. Theoret. Biol.* 2, 201.
- Perl, W. (1976). Analytical approaches to tracer exchange. In *Microcirculation*, (I. Grayson and W. Zingg, eds.), Plenum Press, New York and London.
- Raskin, E.M. (1959). Capillary permeability and transcapillary exchange in relation to molecular size. In *Microcirculation*, (I. Grayson and W. Zingg, eds.), Plenum Press, New York and London, 28-37.
- Sheppard C.W. (1962). Basic principles of the tracer method introduction to mathematical tracer kinetics. New York. Wiley.
- Yudilevich, D. and Martin de Jullin, P. (1963). A method to process the data when several mixtures of elements are to be resolved in their components. Applications to mixtures of  $^{19}\text{F}$ ,  $^{23}\text{N}$ ,  $^{86}\text{Rb}$  and  $^{131}\text{I}$ . *Intern. J. Appl. Radiation Isotopes* 14 563-570.
- Zierler K.L. (1958). A simplified explanation of the theory of indicator-dilution for measurement of blood flow and volume and their distributive phenomena. *Bull. Johns Hopkins Hosp.* 103, 199.
- Zierler K.L. (1962). Theoretical basis of indicator-dilution methods for measuring flow and volume. *Circ. Res.* 11 393-407.



- Zierler K.L. (1962). Circulation times and the theory of indicator-dilution method for determining blood flow and volume. In *Handbook of Physiology* sec. 2 vol. 1 Circulation, (W F Hamilton and P. Dow eds.) Washington, D.C., American Physiol. Society 585
- Zierler K.L. (1963). Theory of use of indicators to measure flow and extracellular volume and calculation of transcapillary movement of tracers. *Circ. Res.* 12, 464
- Zierler K.L. (1965). Tracer-dilution techniques in the study of microvascular behavior. *Fed. Proc.* 24 1085

## MODELS

- Apeiblat, A., Katzir-Katchalsky, A. and Silberberg, A. (1974). A mathematical analysis of capillary-tissue fluid exchange. *Biorheology* 11 1-45
- Basilopkwaighe J.B., Knopp T.J. and Hazelrig, Jane B. (1970). A concurrent flow model for capillary-tissue exchanges. In *Capillary Permeability* (C. Crone and N.A. Lassen, eds.) Munksgaard Copenhagen 60-80.
- Basilopkwaighe J.B. (1974). A concurrent flow model for extraction during transcapillary passage. *Circ. Res.* 35 483-503.
- Blum J.J. (1960). Concentration profiles in and around capillaries. *Am. J. Physiol.* 198(5), 991-994
- Carry F.L. (1974). A hydrodynamic description of the osmotic reflection coefficient with application to the pore theory of transcapillary exchange. *Microvasc. Res.* 8 236-252
- Goresky C.A. (1963). A linear method for determining liver sinusoidal and extravascular volumes. *Am. J. Physiol.* 204 626-640
- Goresky C.A., Ziegler W.H. and Bach, G.G. (1970). Capillary exchange modelling. Barrier-limited and flow-limited distribution. *Circ. Res.* 27 739-764
- Goresky C.A. and Row C.P. (1977). Blood-tissue exchange in liver and heart: the influence of heterogeneity of capillary transit times. *Fed. Proc.* 36 2629-634
- Granger H.J. and Shepherd A.P. (1973). Intrinsic microvascular control of tissue oxygen delivery. *Microvasc. Res.* 5 49-72
- Johnson, J.A. and Wilson, T.A. (1966). A model for capillary exchange. *Am. J. Physiol.* 210, 1299-1303
- Johnson, J.A. (1970). A model for capillary exchange with applications. In *Capillary Permeability* (C. Crone and N.A. Lassen, eds.) Munksgaard, Copenhagen, 206-217
- Johnson, J.A., Levitt, D.G., Wangerssteen O.D. and Bloom, G. (1975). A distributed pore size model for capillaries. *Microvasc. Res.* 10 217-219.
- Kety S.S. (1951). Theory and application of the exchange of inert gas at the lungs and tissues. *Pharmacol. Rev.* 3 1-41
- Krogh A. (1919). The number and distribution of capillaries in muscles with calculations of the oxygen pressure local necessary for supplying the tissue. *J. Physiol. London*, 52, 409-415.
- Leonard J.L. and Abbrecht, P.H. (1973). Dynamics of plasma-interstitial fluid distribution following intravenous infusions in dogs. *Circ. Res.* 33 733-748.
- Levitt, D.G. (1970). Evaluation of the early extraction method of determining capillary permeability by theoretical capillary and organ models. *Circ. Res.* 27 81.
- Levitt, D.G. (1971). Theoretical model of capillary exchange incorporating interaction between capillaries. *Am. J. Physiol.* 220, 250-255
- Levitt D.G. (1975). General continuum analysis of transport through pores. I. Proof of Onsager's reciprocity postulate for uniform pore. *Biophys. J.* 15 533
- Levitt, D.G. (1975). General continuum analysis of transport through pores. II. Nonuniform pores. *Biophys. J.* 15 553
- Lifson, N. (1970). Revised equation for the osmotic transient method. In *Capillary Permeability* (C. Crone and N.A. Lassen, ed.) Munksgaard Copenhagen, 302-305
- Peil, W. (1963). An extension of the diffusion equation to include clearance by capillary blood flow. *Am. N.Y. Acad. Sci.* 108 9-105
- Peil, W. and Chaud, I.P. (1968). A convection-diffusion model of indicator transport through an organ. *Circ. Res.* 22 72-78, 78-503

- Perl, W (1970). An interpolation model for evaluating permeability from indicator dilution curves. In *Capillary Permeability* (C. Crone and N.A. Lassen, eds.), Munksgaard, Copenhagen, 185-201.
- Perl, W (1971). Modified filtration-permeability model of transcapillary transport — solution of the Pappenheimer pore puzzle? *Microvasc. Res.* 3 233-251
- Perl, W (1973). A friction coefficient, series-parallel channel model of transcapillary flux of non-electrolytes and water. *Microvasc. Res.* 6 169
- Perl, W, Silverman, F., Delee, A.C. and Chikard, F.P (1976). Permeability of dog lung endothelium to sodium, dioxin, ureides and water. *Am. J. Physiol.* 230 1708-1721.
- Pollock, F and Blum, J.J (1966). On the distribution of permeable solute during Poiseuille flow in capillary tubes. *Biophys. J.* 6 19-28.
- Prothero, J and Burton, A.C. (1961). The physics of blood flow in capillaries. I. The nature of the motion. *Biophys. J.* 1 564-579
- Rankin, E.M. (1954). Filtration, diffusion and molecular sieving through porous cellulose membranes. *J. Gen. Physiol.* 38, 225
- Rankin, E.M. (1959). Transport of potassium-42 from blood to tissue in isolated mammalian skeletal muscles. *Am. J. Physiol.* 197 1205-1210.
- Saltzer, E.P and Am, K.-N (1976). A mathematical analysis of fluid movement across capillary walls. *Microvasc. Res.* 11, 1-23.
- Sangren, W.C. and Sheppard, C.W (1953). A mathematical derivation of the exchange of a labelled substance between a liquid flowing in a vessel and an external compartment. *Bull. Math. Biophys.* 15 387-394
- Schmidt, G.W (1952). A mathematical theory of capillary exchange as a function of tissue structure. *Bull. Math. Biophys.* 14, 229-263
- Schmidt, G.W (1953). The time course of capillary exchange. *Bull. Math. Biophys.* 15 477-483.
- Ziegler, W.H. and Gorosky, C.A (1971). Transcapillary exchange in the working left ventricle of the dog. *Circ. Res.* 29 181-208
- Wiederholm, C.A. (1968). Dynamics of transcapillary fluid exchange. *J. Gen. Physiol.* 52, 29s-63s.
- Yipiotsof, Knopp, T.J and Baumgartwright, J.B. (1969). A countercurrent exchange model for capillary blood flow. *Fed. Proc.* 28(2), 645.

## REFERENCES ON SOME PREVIOUS STUDIES WITH THE INDICATOR DILUTION METHOD

### Adipose tissue

- Linde, B, Chisholm, G. and Rosell, S. (1974). The influence of sympathetic activity and histamine on the blood-tissue exchange of solutes in canine adipose tissue. *Acta Physiol. Scand.* 92, 145-155

### Bone

- Devies, D.R., Baumgartwright, J.B. and Kelly, P.J (1976). Transcapillary exchange of strontium and sodium in canine tibia. *J. Appl. Physiol.* 40, 17
- Kelly, P.J and Baumgartwright, J.B. (1977). Studies on bone ion exchange using multiple-tracer indicator-dilution techniques. *Fed. Proc.* 36 2634-2639
- Hughes, S.P.F., Davies, D.R., Baumgartwright, J.B., Knox, F.G. and Kelly, P.J (1977). Bone extraction and blood clearance of diphosphonate in the dog. *Am. J. Physiol.* 232, H341-H347
- Hughes, S.P.F., Davies, D.R., Khan, R. and Kelly, P.J (1978). Fluid space in bone. *Clin. Orthop.* 134 332-341.

### Brain

- Agnew, W.F. and Crone, C. (1967). Permeability of brain capillaries to hexoses and pentoses in the rabbit. *Acta Physiol. Scand.* 70, 168.
- Bohner, T.G. and Lassen, N.A. (1975). The diffusion permeability to water of the rat blood-brain barrier. *Acta Physiol. Scand.* 93, 415
- Crone, C. (1961). The diffusion of some organic non-electrolytes from blood to brain tissue. *Thesis* (1. Danish with an English Summary). Munksgaard, Copenhagen.

- Croce, C. (1963). The permeability of capillaries in various organs as determined by use of the "indicator diffusion" method. *Acta Physiol. Scand.* 58, 292-305
- Croce, C. (1965). The permeability of brain capillaries to non-electrolytes. *Acta Physiol. Scand.* 64 407-417
- Gjedde, A. and Croce, C. (1975). Induction processes in blood-brain transfer of ketone bodies during starvation. *Am. J. Physiol.* 229 1165
- Hertz, M.M. and Bohwig, T.G. (1976). Blood-brain barrier studies in the rat: An indicator dilution technique with tracer sodium as an internal standard for estimation of extracerebral contamination. *Brain Res.* 107 333-343
- Yudilevich, D.L. and de Rose, N. (1971). Blood-brain transfer of glucose and other molecules measured by rapid indicator dilution. *Am. J. Physiol.* 220, 841-846.

also Kidney Liver Lung, Skeletal muscle

#### Gastric wall

- Alvarez, O.A. and Yudilevich, D.L. (1967) Capillary permeability and tissue exchange of water and electrolytes in the stomach. *Am. J. Physiol.* 213 315-322.

#### Heart muscle

- Alvarez, O.A. and Yudilevich, D.L. (1969). Heart capillary permeability to lipid-insoluble molecules. *J. Physiol., Lond.* 202, 45-58.
- Durrin, W.N., Alvarez, O.A. and Yudilevich, D.L. (1973). Influence of maximal vasodilatation on glucose and sodium blood-tissue transport in canine heart. *Microvasc. Res.* 6 347-359
- Durrin, W.N. and Yudilevich, D.L. (1978) Estimate of capillary permeability coefficients of canine heart to sodium and glucose. *Microvasc. Res.* 15 195-205
- Goresky C.A. and Rose C.P. (1977). Blood-tissue exchange in liver and heart: the influence of heterogeneity of capillary transit times. *Fed. Proc.* 36 2629-2634.
- Gulley B., Yipintsoi, T. Orris, A.L. and Basingthwaite, J.B. (1975). Myocardial sodium extraction at varied flows in the dog. *Circ. Res.* 37 359
- Laughlin, M.H. and Dumas, J.N. (1975). Myocardial transcappillary exchange in the hypertrophied heart of the dog. *Am. J. Physiol.* 229 838-846.
- Rose C.P. and Goresky C.A. (1976). Vasomotor control of capillary transit time heterogeneity in the canine coronary circulation. *Circ. Res.* 39 541
- Tangredi, R.G., Yipintsoi, T. and Basingthwaite, J.B. (1975). Capillary and cell wall permeability to potassium in isolated dog hearts. *Am. J. Physiol.* 229 537
- Yipintsoi, T., Gustafson, D. and Basingthwaite, J. (1968). Capillary tissue exchange and volumes of distribution of antipyrine in the heart. *Circ., Suppl.* 6 38, 210 (abstract).
- Yudilevich, D. and Martin de Jukin, P. (1965). Potassium, sodium and iodide transcappillary exchange in the dog heart. *Am. J. Physiol.* 208 959-967
- Yudilevich, D.L. and Alvarez O.A. (1967). Water sodium and thionine transcappillary diffusion in the dog heart. *Am. J. Physiol.* 213 308-314
- Ziegler W.H. and Goresky C.A. (1971). Transcapillary exchange in the working left ventricle of the dog. *Circ. Res.* 29 181-207

also Liver

#### Kidney

- Chinard, F.P., Enns, T. and Nolan, M.F. (1962). Indicator dilution studies with diffusible indicators. *Circ. Res.* 10 473-490
- Chinard, F.P., Enns, T., Goresky C.A. and Nolan, M.F. (1965). Renal transit times and distribution volumes of T-1824 creatinine and water. *Am. J. Physiol.* 209 243 252
- Chinard, F.P., Goresky C.A., Enns, T. Nolan, M.F. and Howe, R.W. (1965). Trapping of urea by red cells in the kidney. *Am. J. Physiol.* 209 253 63.

mainly on the Heart-Lung System

## Liver

- Bravo I.R. and Yudilevich, D.L. (1977) rapid indicator-dilution technique  
Goresky C.A., Bach, G.G. and Naeher  
liver. *J. Clin. Invest.* 52, 975-984  
Goresky C.A., Bach, G.G. and Naeher  
effect on the exchange of material

- and uptake of molecules studied by  
1449-1453  
on the uptake of materials by the intact  
and cell carriage of label its limiting ef-  
36 328-351

## Lung

- Basset, G. Flez-Vaschal, P.Y. F.D.  
pulmonaire par la methode d  
Europ. Soc. for Microcirculat  
Brigham, K.L. and Snell, J.D. (19  
pulmonary edema. *J. Clin. Inv*  
Chinard, F.P. Erms, T. and Nolu  
tangent time and slope data. *J. Appl Physiol.* 17, 179-183  
Chinard, F.P. (1966). The permeability characteristics of the pulmonary blood-gas barrier. In *Advances in Respiratory Physiology* (C.C. Caro ed.) Baltimore, Williams & Wilkins, 106-147  
Effros, R.M. and Chinard, F.P. (1969). The in vivo pH of the extravascular space of the lung.  
*J. Clin. Invest.* 48, 1983-1996  
Friebel, N., Schreiner, G.E. and Athem, J.W. (1952). A new method for measuring transcapillary  
exchanges: the transfer of salt and water in the lower circulation of man. *J. Clin. Invest.* 31  
629 (Abstr.)  
Goresky C.A., Crochin, R.F.P. and Wang, E. (1969). Indicator dilution measurements of  
extravascular water in the lungs. *J. Clin. Invest.* 48, 487-501  
Gomp, F.E., Meshima, Y., Jazgon, S. and Kinney J.M. (1971). Simultaneous use of three indi-  
cators to evaluate pulmonary capillary damage in man. *Surgery* 70, 262-270.  
Harrie, T.R., Rowlett, R.D. and Brigham, K.L. (1976). The identification of pulmonary capillary  
permeability from multiple-indicator data. *Microvasc. Res.* 12, 177-196.  
Lindenfeld, L.S., Fries, E.D., Partonope, E.A. and Morawitz, H.J. (1935). Transcapillary migration  
of heavy water and thiocyanate ion in the pulmonary circulation of normal subjects and pa-  
tients with congestive heart failure. *J. Clin. Invest.* 34, 1-11.  
Newfield, G.R., Williams, J.J., Graves, D.J., Soane, L.R. and Marshall, B.E. (1975). Pulmonary ca-  
pillary permeability in man and canine model of chemical pulmonary edema. *Microvasc. Res.*  
10, 192-207  
Pearce, M.L. (1969) Sodium recovery from normal and edematous lungs studied by indicator di-  
lution curves. *Circ. Res.* 24, 815-820.  
Puri, W., Silverman, F., Delea, A.C. and Chinard, F.P. (1976). Permeability of dog lung endothel-  
ium to sodium, diuretics, anions and water. *Am. J. Physiol.* 230, 1703-1721.  
Yipintol, T. (1976). Single-passage extraction and permeability estimation of sodium in normal  
dog lungs. *Circ. Res.* 39, 523-531.  
Yudilevich, D.L. (1961). Transport of  $K^{40}$ ,  $N^{15}$  and  $I^{131}$  by the pulmonary circulation. Calcu-  
lations of cardiac output and volume from dilution curves obtained in the canine heart-lung  
preparation. *Circ. Res.* 9, 925-935

## Placenta

- Yudilevich, D.L. (1976). Transport across capillary and cellular barriers of the placenta studied by  
single circulation multiple-tracer dilution. *J. Physiol.* 256, 84-85  
Eaton, B.M., Lechtwiler, H.P., Short, A.H. and Yudilevich, D.L. (1979). A isolated guinea-pig  
placenta for the study of transport by rapid paired-tracer dilution. *J. Physiol.* (In press).

## Salivary gland

- Mason, G.E., Smaje L.H. and Yudilevich, D.L. (1976) Vascular permeability in the perfused cat  
salivary gland using single passage multiple tracer dilution. *J. Physiol.* 258, 58-60  
Mason, G.E. (1979). Capillary and epithelial exchange in the cat submandibular gland. Doctoral  
thesis, Faculty of Science, University College, University of London

#### Single capillary (mesenteric)

- Croce, C. and Friedman, J.J. (1976). A method for determining potassium permeability of a single capillary. *Acta Physiol. Scand.* 96 13A-14A.
- Croce, C., Frekjaer Jensen, J., Friedman, J.J. and Christensen, O. (1978). The permeability of single capillaries to potassium ions. *J. Gen. Physiol.* 71 195

#### Skeletal muscle

- Aberg, B. (1973). Restricted convection-diffusion across capillary walls. Doctoral thesis. *Acta Univ. Upsalensis* 160, 1-36.
- Croce, C. (1973). Transcapillary transport of D- and L-glucose in isolated skeletal muscle. *Acta Physiol. Scand.* 87 138
- Fleming, B.P. and Dias, J.N. (1977). Interaction between fluid movement and solute exchange in the isolated dog hindlimb. *Biol. Anal.* 15 483-488
- Freis, E., Higgins, T.F. and Morawitz, H.J. (1953). Transcapillary exchange rates of dinitrobenzyl oxide and thiocyanate in the forearm of man. *J. Appl. Physiol.* 5 526.
- Friedman, J.J. (1969). Single passage extraction of  $^{86}\text{Rb}$  from the circulation of skeletal muscle. *Am. J. Physiol.* 216(3) 460-466.
- Garlick, D.G. (1970). Factors affecting the transport of extracellular molecules in skeletal muscle. In *Capillary Permeability* (C. Croce and N.A. Lassen, eds.) Munksgaard, Copenhagen, 228-238
- Pease, W.P. and Sejvar, P. (1977). Transcapillary exchange of 14-C-inulin by free diffusion in channels of fused vesicles. *Acta Physiol. Scand.* 100 437
- Rippe, B. and Stage, L. (1976). A colorimetric method for repeated, rapid determinations of capillary diffusion capacity. *Acta Physiol. Scand., Suppl.* 440.
- Rippe, B., Kamiya, A. and Folkow, B. (1978). Simultaneous measurements of capillary diffusion and filtration exchange during shifts in filtration-absorption and at graded alterations in the capillary permeability surface area product (PS). *Acta Physiol. Scand.* 104 318-336.
- Rippe, B. and Grega, G.J. (1978). Effects of isoprenaline and cooling on histamine induced changes of capillary permeability in the rat hindquarter vascular bed. *Acta Physiol. Scand.* 103 252-262.
- Trap-Jensen, J. and Lassen, N.A. (1970). Capillary permeability for smaller hydrophobic tracers in exercising skeletal muscle in normal men and in patients with long term diabetes mellitus. In *Capillary Permeability* (C. Croce and N.A. Lassen, eds.) Munksgaard, Copenhagen, 135-152.
- Trap-Jensen, J. and Lassen, N.A. (1971). Restricted diffusion in skeletal muscle capillaries in man. *Am. J. Physiol.* 220 371
- Yodanis, D.L., Renkin, E.M., Alvarez, O.A. and Bravo, I. (1968). Fractional extraction and transcapillary exchange during continuous and instantaneous tracer administration. *Circ. Res.* 33 335-336

also Gastric wall, Liver

#### Spleen

- Canard, F.P., Vosburgh, G.J. and Eans, Th. (1955). Transcapillary exchange of water and of other substances in certain organs in the dog. *Am. J. Physiol.* 183 221-234

also Brain, Kidney, Liver, Skeletal muscle





**Acta Physiologica Scandinavica**  
**Supplementum 473**

**XVI Scandinavian  
of Physiology and  
Oulu 1979**

**Abstracts**

**Oulu 1979**





**XVI Scandinavian Congress  
of Physiology and Pharmacology  
Oulu 1979**

**Abstracts**

Printed in Finland

Kirjapaino Osakeyhtiö Kakeva - Oulu - 1979

# SUBJECT INDEX

INVITED LECTURES	Abstract number
Cardiac performance	1
Reflex control of the circulation during exercise	2
Regulation of prolactin (PRL) secretion and synthesis	3
Neuropeptides and behaviour	4
Information processing in neural systems	5
Neurophysiology of mentality	6
Hypothalamo-pituitary regulation of liver metabolism	7
Mechanism of adverse drug reactions	8
Stress defence and coping in man and animals	9
Taurine in platelets and heart tissue	10
Benzodiazepine receptors and their possible endogenous ligands	11
Renal homeostatic responses	12
Local factors in the regulation of interstitial fluid volume	13
SYMPOSIA	
Central control of circulation	14—17
Multiple peripheral sources and effects of biologically active peptides	18—22
Biological significance of extrahepatic drug biotransformation	23—28
Sensory transduction processes	29—32
Biological effects of alcohol	33—37
Experimental animals in research	38—41

## COMMUNICATIONS

Cardiovascular system	42—78
Exercise    Skeletal and smooth muscle	79—97
Respiration	98—104
Gastrointestinal tract	105—118
Kidneys    Body fluids	119—133
Endocrinology    Peptides	134—153
Thermoregulation	154—160
Nerves and neurons	161—178
Sensory system	179—187
Sleep	188—195
Central nervous system pharmacology	196—215
Pharmacokinetics	216—236
Drug actions	237—261
Toxicology	262—275
Biotransformation	276—281

1

**III. Viscosity** (Institute for Experimental Medical Research, University of Oslo, Norway); **GAUJARD PERFORMANCE**

A main goal of our studies on both conscious and anesthetized open-chest dogs has been to apply to the intact heart concepts derived from studies on isolated papillary muscles. An ultrasonic technique for measurement of cardiac dimensions has been extensively used by many investigators; during ejection the diameter of the ventricular cavity is reduced whereas the myocardial wall thickness. This technique can therefore not be used for estimations of fibre shortening. I obtain more direct estimates of variations of mean changes in myocardial fibre length, ultrasonic elements were sutured into the anterior wall of the left ventricle centimetre apart and the distance between them, the myocardial chord length (MCL) continuously recorded. When the maximal MCL coincides with start of ejection and minimal MCL with completion of ejection, the recordings are representative for the whole myocardium; high correlation was obtained between stroke volume and the differences between end-diastolic and end-systolic chords estimated from MCL recordings. Such recordings have led to the following conclusions:

1. A rise in afterload (induced by proximal aortic constriction) reduces myocardial contraction so that end-systolic MCL increases, but is without effect on end-diastolic MCL. Afterload may also be increased by ventricular septation at constant systolic left ventricular pressure. The increase in end-systolic MCL caused by increased afterload is large at low isotropy and may not be significant at high isotropy. There is no sign of "homeostatic autoregulation".
2. A rise in preload results in increased end-diastolic MCL and may reflect increased circulating blood volume during elevation of systemic blood pressure. Rise in preload is consequence of redistribution of blood volume towards the heart. Decrease of increased end-diastolic volume and almost unchanged end-systolic volume. Rise in systemic blood pressure may at high

- and constant isotropy increase stroke volume.
3. Variations in heart rate between 40-80 beats/min have no effect on end-diastolic MCL and stroke volume. A rise in heart rate above 80 beats/min reduces end-diastolic MCL because of curtailed filling, but exerts little effect on end-systolic MCL. A rise in blood volume increases stroke volume more at low than at high heart rate. When control end-diastolic volume has been restored by raising heart rate, control end-systolic volume is also restored. A rise in heart rate exerts therefore no isotropic effect; variations in heart rate and blood volume affects stroke volume only by altering preload.
4. A rise in isotropy reduces end-systolic MCL but has no effect on end-diastolic MCL if heart rate is kept constant. The reduction in end-systolic MCL and increase in stroke volume induced by raising isotropy is equal at any heart rate.

Thus, rise in isotropy reduces end-systolic volume and is without effect on end-diastolic volume. Conversely, rise in preload induced by increasing circulating blood volume or reducing heart rate towards 80 beats/min, increases end-diastolic volume but exerts little effect on end-systolic volume. Therefore, isotropy and chronotropy are independent variables in the regulation of stroke volume.

2

**ROUILL, J.-J.** (Departments of Physiology and Biophysics and Medicine University of Washington School of Medicine Seattle WA) **REFLEX CONTROL OF THE CIRCULATION DURING EXERCISE**

Human circulatory responses to exercise are now well documented. But the stimuli, the effect (A) the lower matching heart rate (HR) and cardiac output (CO) to  $\dot{V}_{O_2}$  (B) the rise in arterial blood pressure (BP) and (C) vasoconstrictions in non-working regions are still unknown. The following have been postulated to provide the exercise stimulus (1) sensory inputs released from working muscle (2) arterial baroreceptors (3) arterial chemoreceptors (4) central venous chemoreceptors (5) irradiation of nerve impulses from the motor cortex (central command) (6) muscle mechanoreceptors and (7) muscle chemoreceptors. With (1) (4) above previously ruled out, recent attention has focused on (3) (7). Central command (3) probably plays a lesser role. Studies employing differential nerve blockade and nerve recordings (in cats and dogs) support the muscle mechanoreceptors (6) play no role. Since 1896 when Renshaw and Osborn first postulated their role, investigators have tested the idea that muscle chemoreceptors (7) could provide the "exercise stimulus" by emitting certain chemical changes within the muscle. The early demonstrations by Allen, Buick and Arnesen, Halpern that pressure responses accompany contraction of ischemic muscle form the keystone of this thinking. The pressure responses associated with ischemic exercise may be related phenomena. In man and other species interruption of sensory nerve from contracting muscles will abolish this response which in cats appears to be carried by the fibers.

Recent efforts to characterize further the extent muscle pressure responses (DPR) led by 10-30 sec periods of leg occlusion during exercise with occlusion continued for 3 min after exercise showed the following during 3 min of post exercise occlusion: (a) DPR is graded in direct proportion to the degree of muscle

ischemia and also (b) is directly proportional to the ischemic muscle mass. (c) DPR usually results from higher than normal  $\dot{V}_{O_2}$  during occlusion. (d) DPR is accompanied by marked vasodilatation (vasodilation) in areas where  $\dot{V}_{O_2}$  and HR are reduced. DPR is maintained by vasoconstriction. (e)  $\dot{V}_{O_2}$  and end-tidal  $\text{CO}_2$  are unaffected by post exercise muscle ischemia. (f) DPR is increased by neuromuscular fatigue (increased sensitivity) (increased thoracic blood volume) and reduced by neuromuscular fatigue (decreased thoracic blood volume) (4) differential blockade of sensory nerves (peripheral anesthesia) abolishes DPR but does not block normal cardiovascular respiratory responses to exercise without occlusion.

These above results support that (1) the baroreflex helps to set the lowered level of HR during DPR. (2) There is no drive from muscle chemoreceptors to respiratory centers. (3) Normal exercise responses may not require functioning -fibers or alternatively (4) large (unblocked) nerve fibers may also conduct muscle chemoreflexes or (5) small chemoreflexes may only be activated when muscle perfusion falls below some critical level and then they somehow raise the baroreflex operating point.

PRL is a polypeptide hormone which is synthesized and secreted by anterior pituitary cell T. To study the function of lactotrophic cells we have used a clonal strain of rat pituitary tumour cell which spontaneously secretes PRL *in vitro*. These cells respond to the hypothalamic releasing factors TRH and to TRH-antiserum dual. Both hormones stimulate PRL production but they exert their effect through different molecular mechanisms. TRH induces half maximal stimulation of secretion and synthesis at 0.3 nM and 3.0 nM respectively. The following parameters have been measured and are believed to be of importance for initiating the biological effect: 1)  $^{125}$ I-TRH binds rapidly to the cells at 37°C and 2/3 of maximal binding occurs within 10 sec. The binding of  $^{125}$ I-TRH is specific (saturable) and reversible. 2) Maximal binding equals 50-400 fmoles  $^{125}$ I-TRH/cell per 10<sup>6</sup> cells. 3) No different high affinity binding sites with apparent  $K_d$  of 0.3 nM and  $K_d$  of 25 nM are present. 2) Electrophysiological studies show that these cells exhibit spontaneous action potentials whose duration and frequency are increased after addition of TRH. 3) Addition of TRH to monolayer cultures causes transient increases (2-5 fold) in cellular cyclic GMP and in cyclic AMP (2-3 fold). The concentrations of TRH that are needed for half maximal stimulation of cGMP and cAMP elevations are about 3 nM and about 0.05 nM, respectively. 4) Nicotinic esterase inhibitors (thiocholine, acetylthiocholine) stimulate secretion as well as synthesis of PRL and increase the concentration of cyclic nucleotides. 5) Fluoride cyclic AMP and cyclic cyclic GMP stimulates the synthesis of PRL.

but only dibutyryl cyclic AMP is able to enhance PRL secretion. 6 Cells cultured in calcium free medium show reduced spontaneous secretion of PRL and TRH is unable to stimulate hormone secretion. When cyclic nucleotides are measured after TRH treatment in the absence of calcium, cAMP elevations are still observed but no increase in cGMP formation occurs. 7 After pre-loading of the cells with  $^{45}\text{Ca}$  TRH induces an increased efflux preceding PRL secretion. 8 The cell contains two different cAMP phosphodiesterase activities with  $K_m$  of 0.7150  $\mu\text{M}$  and of 20.2  $\mu\text{M}$  respectively. Treatment of the cells with TPI increases 50% of both enzyme activities with time-course similar to that of cAMP elevation. 9 In cell homogenate cAMP dependent and independent protein kinase activities are demonstrated. The cAMP dependent protein kinase is half maximally stimulated by TRH at about 25 nM. 10 The binding of 44-178-estradiol to 30000-year-old cultures of  $^3\text{H}$  is rapid (half maximal at 2 hr), saturable, specific and reversible. 44-178-estradiol radioactivity accumulates in cytoplasm at  $4^\circ\text{C}$  but is translocated into the nucleus when the temperature is increased to  $37^\circ\text{C}$ . The cytoplasmic radioactivity is found to be associated with macromolecules sedimenting in sucrose density gradients as 5S substances and with nuclear 5S macromolecules. 11 The combined treatment of TRH and 178-estradiol shows additive effects on PRL synthesis. 12 TRH pretreatment reduces the number of binding sites for 44-178 as well as the TRH-induced elevation of cAMP while 178-estradiol pretreatment doubles the number of available 44-178 binding sites. 13 The mode of action of TRH and 178-estradiol on PRL synthesis is studied further in cell free wheat germ translation system. Possible changes in PRL specific mRNA activity associated with stimulated hormone synthesis are recorded and compared to the importance of alterations in cellular receptor sites for PRL induction after treatment with TRH and 8-estradiol.

Neuropeptides have dual role in relation to behavior processes: (a) as hypothalamic hormones link between environmental stimuli and the secretion of endocrine hormones that control behavior and (b) possibly directly on neuronal mechanisms involved in behavior.

The present study was designed to investigate the relationship between the degree of social support and the level of self-esteem in the context of a group therapy program. The results showed that there was a significant positive correlation between the two variables.

1 The pacifi ffer on spont seou beha io  
1 beh i bei g normal cons itue t f  
the inal spe are uch as feeding so-  
i i i ion vnal behavi

2 The model try to see if drug-induced  
behaviors in the rat are self-reinforcing  
effects but it is not inhibitory the  
power of psychotropic drugs

1 The effect on the road surface was  
 di have been led on so lid me  
 9 tively condit ioned bet 1 The animal  
 1 rn o void neg ve ff by pr  
 di 1 1 cert 1 mul 4e cpep id

have bee f und t i five diff f  
of thi f oce  
Chelery told i ha been one day  
food t t m t t t t t d h ng  
d LFFP reportedly f t t ce lomeris

peptide whi 4 mod 1 d 7 nd cel beha  
i re i TSP H.P I wh ch f il  
tal vapor as E DPA T i now te ve  
dome tation hwi 3 tr pept de c

opr 1 MSH/ACTH 1 Tm 3 14 1  
thing 11 11 no 3 re 1m y w ch

suggest that neuropeptides might influence behavior by acting on processes which are more common to a large part of the machinery producing behavior. Certain general peptide structures might be found to have selective action on some processes related to vigilance and attention. Behavior is dependent on contact between the individual and specific environments. The ability to direct mental activity must be very essential pre requisite for the further promotion of certain behavior. Learning and memory activities are also the possible tool for the action of such peptide. This is of course of obvious significance for behavior in particular situations. The integrated function of innate and acquired mechanisms. Any difficulty to detect recognition to consolidate recall and drive if reaction should be very important for behavior.

The effect of  $\beta$ -endorphin on exploratory and  
social-real behavior patterns will be described  
and discussed with regard to what is known  
above.

I pit of the modern very sensitive th-  
al i y the ignifi ne II neuro peptide  
for mental ctivi y must leo be based on r le-  
vs function l me sur Incr ed i l q h i t  
th chun l e l b e x i n g f o r p e p t i d e s

ly defi ed behavi l iters be su ed (f  
f new aif the di ca lon ee Mays on  
B J Hypothalavi Hormone d Beh lo Medi

1 JUL 1979 1 5

Bergström, R.M. (Institut f Physiologi  
University f Helsinki Finland) INFORMATION  
PROCESSING IN NEURAL SYSTEMS

The abstract statistical information theory takes no account for the conservation of energy and matter in the communication system. This might be the use of its lack of success in explaining neurophysiological processes, especially higher order information processing. An alternative is the physical information theory (Brillouin, L. Science and information theory New York, Academic Press 1963) which connects information flow with flow of entropy and distribution of energy and matter. In neural systems processing of information is proceeding by distributions of signals and their material substrates the synapses.

Whereas the coding mechanisms in single neurons are rather well known, there are great difficulties concerning complex networks. This is considered as being due to lack of knowledge of the wiring geometry of microcircuits (Edelman, G.M. and V.B. Mountcastle. The wandering brain Cambridge MIT Press 1978). However, this geometry is not stable, significant changes are needed for description of variations in synaptic plasticity, inhibition etc. together with variation of signal activity (code parameters being number of units (N), number of hierarchical synapses (M), amount of signals (I) and their order (j)). Attempts for pacification of such magnitudes have been made (Scott A.C. Neurophysiology John Wiley & Sons Ltd 1977). These magnitudes may be of the main form  $Q \propto N^j M^k I^l$  defining the effect of neurons with M synapses and average spike frequency  $Q$ . It is possible to define concept of an neural microstate (of elements) and macrostate (of averages) with corresponding levels.

Information processing (Prigogine I Physics Today 1977 28 23). As an example, an integration of microstates over M neurons defines macrostates of which the entropy distribution (I) and hence the information capacity possesses maximum at medium level of average spike frequency.

There also exists the possibility of defining the neural macrostate as subjective psychological state (measurable only with psychological methods) and the neural microstate as an objective physiological state (measurable only with physiological methods) (Bergström R.M. In Advances in Psychobiology (Eds G. Newton and A.M. Riesen) John Wiley 1977). As predicted by this definition, the calculation of average of elementary neural events should reveal the same mathematical information functions, the direct measure of the corresponding psychological states. As an example of his initial parabolic dependence (inverted U-shaped functions) of information processing capacity on the level of excitation can be obtained both by calculation from the physiological (microstate) parameters of the neural system (Bergström R.M. and Nevalainen O. In: J. Neurosci 1978 4 171) and by direct psychological (macrostate) measurement (Webb D.O. The organization of behavior Wiley New York 1963).

The definition of higher order conscious information processing as macrostate processing possesses no difficulties from the thermodynamic point of view as regards the interaction between physiological and psychological states. For the solution of the mind-brain problem inherent in this view, loaded with difficulties contained in the methodological phenomenological parallelism and identical solutions.

Rechtschaffen, H.P. (Department of Human Neurophysiology, Institute for Experimental Medicine Acad. Med. Sci. USSR, Leningrad USSR) NEUROPHYSIOLOGY OF MENTALITY

When studying the multimodal activity of neuronal populations of the human brain (in the course of diagnosis and therapy) with the aid of original, adapted and standard mathematical technical approaches, the areas were revealed in which perception and reproduction of words were correlated with local spatial-temporal rearrangement of the multimodal activity - the patterns. The semantic component of words is characterized by presence of common element in their patterns, the group sequence of discharges with fixed intervals. The specific character of the patterns and of some of their elements suggested the standard computer search technique using the former as standards. Accumulation of individual banks of the patterns-standards for different words made it possible to study the neurophysiological dynamics developing during the long-term memory activation in the course of generalization, mental conclusion, decision making. The local patterns in multimodal activity are virtually different in different links of the system for maintenance of mental activity which indicates the spread of the neurophysiological organization of mentality and unequal contribution of different brain structures in these phenomena. Local patterns of the same word in different cerebral areas and patterns of words belonging to the same semantic field in the same area possess also certain common element which seems to be neurophysiological expression for system-forming factor.



Quest f soc, J A (Depa tments f Chemi try and Medi l h t ition f Rolinska Institute Swede )  
HYPOTHALAMO-PITUITARY REGULATION OF LIVER METABOLISM

Sex diff renc exi t i hepati drug and te-roid metabolism in the rat nd pos ibly i a number of other species including man Sex diff erces in drug and roid metabolism a not seen before 30 days f age but the ignal fo the diff erces i give soon afte birth Thi ignal i thought t be t test runs t lee t partly acting vi conversion to estroy a on n imprinting cent i the H A

The press ion f the imprinting i medi ted via the pituitary gland which only in the f mal t acret fact (t read femi i ing fact ) that maint ins the femal type metabo- li pattern The effect f femi i ing facto i i m t on hepati cytochrome P-450 Sex t roid in the adult pe iod effect hepati te roid and drug metabolism f a effect on femi i ing fctor secret ion The nat me f thi hypophyseal factor i yet unknown but it does not appear t be one of the known pituitary hormones

The imprinting ce tr ha been shown to re side in the hypothalamus Frontal d ff enta tion t the trochi smati and suprachi smati level wit i correl t femi i tion of hepati t roid metabolism i mal ts Such an effect i lso seen when i ion involv ing mal ly the sat lor pe i ent icula hypothalam and the supr bi smati nucleus re per- formed i mal t No effect re seen in as loqu i lions i femi t i ny f the es todied

The finding sugg t that egion involv ing the t lor hypothalam periventricula the suprachi smati nucleus and adjacent

are is involved in the control of hepati E roid metabolism It i post lated that the neuro- ncal cell bodie that produce a factor with an inhibitory effect on the secret ion f femi i ing factor have their origins in the reat of the hypothalamus or alternatively, may send axons through this re to the basal hypothala- mus and thus directly o indirectly influence the ante lo pit itary gland

With liver membranes from femal rats, 6 H of totally added <sup>125</sup>I hPrl i specifically bound whereas in mal live memb no fractions the specific Prl binding i very low o absent Ante- lor hypothalamio deaff restation at the retro- chi smati level in mal f ts increase the hepati hPrl receptor concs tr tion to the femal level 3 day following the ope tion A tran section rostr l to the suprachi smati nucleus ha no effect on the concentr tion of hPrl re- ceptor i male animal These its democ- tr te that the number f hPrl recept re i regu- l ted by the hypothalamo-pit it ry system The receptor induv ing pituitary factor might be re- l ted i the femi i ing factor

Schroy, J (Dep tment f Pharmacology  
Unve ity f Copenhagen 2 J lians P i  
Vej DK 2100 Copenhagen Ø) MECHANISMS OF AD-  
VERSE DRUG R ACTIONS

Adve se drug ction covs all ction whi h un ntcd i re pect ve f thei ch ct They may i be well known toxic re ction an expected and ly occur i g action i hout pe if dose dependency As ogge t d by Mitchell t i they an be di id d i i Exogge ed unvant d drug ction 2 T i d ug ct on cell cel drug d ced acopi i d drug il gy d f lly one 3 D ug ction f unknown logy Spe i l focu i l i te on t i on h p be wee phy sco hemi i ct ct i i and th occu ence f d ff ct High lipid i b l y (HL) re ry f drug att ent ve t CNS but PLB i ffi l y il hr i p d lubl conf tment the body d low ren i i ce d u t ut i b g on con equence No PLB ence ave gh ffi lity t t live d y boll ed ith or bl r Tr d f net bolit and g oblen f f f ll ill be di cu ed I re tally long t rs ct e- genl y cu gen lity d g oduct on i lly t ve been gl en n more th g be J y i c l l r t f il le given t i th l i f f y be t i i bef i c m f ve f ut e J upon t cu ne h d f f diet ve troloids i l g on Enal J R chell W il ers

Gill tt Toxic Drug Reaction in Concept i  
Biochemi l Pharmacology Eds Gill tt i  
Mitchell 5p iage Ve lag Be ll 1975





Ankleid, K (Institute of Physiology University of Bergen Norway) LOCAL FACTORS IN THE REGULATION OF INTERSTITIAL FLUID VOLUME

As long as the intracellular volume remains constant the total extracellular volume (ECV) affects the balance between intake and renal excretion of salt and water. However, the partition of ECV between plasma and interstitium is governed by net capillary filtration and lymph flow allowing changes in local interstitial fluid volume (IFV) in the face of unchanged total ECV. Examples are orthostatic edema and acute lung edema.

In the case of increased microvascular pressure  $P_c$  in passive depending limb the rise in capillary pressure ( $P_c$ ) and net filtration pressure ( $\Delta P = P_c - P_i - \pi_p + \pi_i$ ) where  $P_i$  is interstitial fluid hydrostatic pressure,  $\pi_p$  and  $\pi_i$  coll osmotic pressures of plasma and interstitial fluid) will increase net filtration ( $F$ ) and IFV. However several local mechanisms will tend to counteract edema formation and one may envisage three lines of defense (I-III).

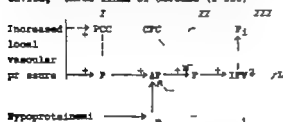


Fig 1. and Increase and decrease of interstitial fluid volume (IFV) and edema-opposing mechanisms

Henneryn, G. and Öberg, B. (Departments of Physiology and Medicine, University of Göteborg, Göteborg and Department of Physiology, College of Veterinary Medicine, Uppsala, Sweden) CIRCULATORY ADJUSTMENTS INDUCED FROM THE VENTRAL SURFACE OF THE CAT MEDULLA OBLONGATA.

Cardiovascular effects elicited by bilateral local cooling of and topical application of drugs on restricted areas on the ventral surface of the medulla oblongata corresponding to the caudal part of the rostral rhombencephalon (Mitchell et al. 1963) and the intermediate area (Schiffke and Loebschke 1967) have been studied in chloralose-anesthetized cats (Henneryn and Öberg, in press).

Cooling of or topical application of GABA or glycine on these structures resulted in a marked reduction of the kidney and the skeletal muscle blood pressure flow and the depression of the respiratory flow. Reflex responses except for a slight increase in heart rate instead of a lowering occurred with application of physostigmine.

During application of the inhibitory transmitter substance GABA withdrawal of arterial baroreceptor restraint (carotid occlusion) produced only very small and markedly diminished increase in peripheral resistance in the renal vascular bed while carotid occlusion still led to substantial vasoconstriction in the skeletal muscle vascular bed. Arterial baroreceptor stimulation during topical GABA application induced initially no further vasoconstriction in the renal vascular bed while marked vasodilatation was still produced in the skeletal muscle vascular bed. These findings suggest that GABA application leads to a general diminution and that the kidney initially complies to cessation of the tonic vasoconstrictor fibre activity.

Topical application of methyl tropine leads induced blood pressure rise and an augmentation of vasoconstrictor fibre tone in renal and skeletal muscle vascular beds. After methyl tropine application the reflex vaso-

I Through myogenic and/or local nervous afferent mechanism the rise in vascular pressure will induce precapillary constriction (PCC) and thereby reduce effective capillary surface area and filtration coefficient (CFC) and diminish the rise in  $P_c$ . Both effects will oppose the rise in net capillary filtration ( $F$ ).

II The second line of defense is activated by the increase of  $P_c$  and IFV. A rise in capillary filtration and dilution of interstitial protein will reduce  $P_i$  and the increase of IFV will increase  $P_i$  with resulting fall in  $\Delta P$ . The relative magnitude of changes in  $P_i$  and  $\Delta P$  will depend on interstitial compliance. A fall in  $\Delta P$  seems most important in skin muscle and intestine.

III Lymph flow (L) will increase in response to rise in  $P_i$  (and possibly IFV). Besides removing water rise in L is necessary to maintain low  $P_i$  by removal of protein.

Mechanism I is immediate and independent of changes in IFV and I probably the most important is everyday orthostasis. Mechanism II is slower, developing in the course of hours. The part played by reduction in  $P_i$  provides only transient protection unless  $P_i$  is increased (III). Only mechanisms II and III are activated in hypoproteinaemia and possibly also by chronically elevated venous pressure (e.g. congestive heart failure). Though the II and III may oppose at least 10 mm Hg fall in  $P_c$  increase in  $P_c$  with contraction of total ECV the same mechanisms tend to retain fluid in the interstitium. Thus the plasma volume is not maintained unchanged at the expense of IFV. Teleologically the resulting change in plasma volume represent the necessary signal to reflex control of renal water and salt excretion.

With contraction of total ECV the same mechanisms tend to retain fluid in the interstitium. Thus the plasma volume is not maintained unchanged at the expense of IFV. Teleologically the resulting change in plasma volume represent the necessary signal to reflex control of renal water and salt excretion.

constrictor response of the kidney to removal of arterial baroreceptor restraint was drastically potentiated while that in the skeletal muscle vascular bed was less affected.

The baroreceptor structures seem to play an important role for the maintenance of the tonic vasoconstrictor fibre activity, especially that in the kidneys. It seems as if the degree of engagement of the renal vascular bed in the arterial baroreceptor reflex is particularly sensitive to changes in influences from the skeletal structures on the ventral medulla while the skeletal muscle vascular bed seems less affected. The observations in the baroreceptor reflex response patterns obtained in these experiments upon drug application on these areas on the medulla surface largely resemble the variations in baroreceptor reflex response patterns which are observed during hypo- and hypercapnia respectively.

#### References

- Mitchell R.A., R.H. Loebschke, J.W. Severinghaus, R.V. Richardson and W.H. Moulton. *Ann. N.Y. Acad. Sci.* 1963 109: 661-681.
- Schiffke, R. and R.H. Loebschke. *Physiologia Arch.* 1967 237: 281-270.

Henneryn G. and B. Öberg. *Physiologia Arch.* 1979, in press.

Svensson, T.H., Engberg, O. and Thordh, P. (Departments of Pharmacology and Physiology, University of Göteborg, Sweden)  
**ACTIVITY OF BRAIN NORADRENERGIC NEURONS IN SPONTANEOUSLY HYPERTENSIVE RATS**

Noradrenergic neurones in the pontine nucleus locus caeruleus (LC) have been implicated in the mediation of arousal and sensory reactions as well as in the regulation of autonomic cardiovascular function. Thus electrical stimulation of the LC has been found to produce pressor response which however was reduced in spontaneously hypertensive rats (SHR). Moreover afferent stimulation of the vagus nerve was found to cause inhibition of LC neuronal activity. We have used single cell recording techniques to further characterise the LC function in chloral hydrate anaesthetized SHR. The average firing rate of randomly encountered LC noradrenergic neurones was progressively reduced with increased age and blood pressure of SHR when compared with normotensive age-matched Wistar Kyoto control rats (WKE). Bilateral vagotomy in the neck did not affect the reduction in LC neuronal firing rate. The activation of LC noradrenaline-containing neurones by yohimbine, an  $\alpha_2$ -receptor antagonist, was more pronounced in SHR than in WKE suggesting deficient autoreceptors function in the LC of SHR. These receptors seem to mediate noradrenergic autoinhibitory influence from even collaterals which can serve to dampen the impact of repetitive stimulation of LC neurones. In biochemical experiments the in vivo rate of tyramine hydroxylation was found to be equal in LC neuronal activity of SHR is depressed below normal in anaesthesia in parallel with their cardiac output and heart rate. The NA neurones in the LC of SHR may consequently be particularly sensitive to environmental stimuli.

(Supported by the Swedish Medical Research Council project nos. 4747, 4764 and 0016.)

Johnson, L. (Department of Physiology College of Veterinary Medicine, Iowa State University, Ames, IA 50011)  
**CENTRAL CONTROL OF BLOOD PRESSURE: POSSIBLE ROLE OF HISTAMINE**

The role of noradrenergic and serotonergic systems in the central control of blood pressure has been the subject of much investigation. Also adrenergic, cholinergic, and dopaminergic neurons are known to participate in this control but to a precise role is not yet clear.

I review the present body of evidence for possible roles of these systems in the central control of blood pressure. (See Johnson, L. (1977) 17-325) The serotonergic system is involved in the central control of blood pressure. The serotonergic system is involved in the central control of blood pressure. The serotonergic system is involved in the central control of blood pressure.

Hi tamine has been found to affect various functions in the brain. In 1957 Trendelenburg (Circ Res 5: 105) showed that low doses of hi tamine (1-10 mg/kg) in the rat produced a decrease in blood pressure. This effect was probably mediated by the central action of hi tamine. The effect of hi tamine could be blocked by the  $H_1$  receptor antagonist, mepyramine. The effect of hi tamine on blood pressure was modified by the  $H_2$  receptor antagonist, cimetidine. It has been suggested that the effect of hi tamine on blood pressure is mediated by the  $H_1$  receptor.

I have studied animal hi tamine given centrally can tentatively increase the heart rate where in conscious animal the heart rate does not change. Decreases in blood pressure have been reported to be low even after blood pressure has been normalized (Hoffman & Schmidt, Life Sci 1978, 22: 1709).

The availability of selective hi tamine  $H_1$  and  $H_2$  agonists has made it possible to study these receptors separately. Injection of  $H_2$  agonist, dimaprit, has been shown to lower blood pressure and heart rate in urethane-anesthetized rats and these effects are blocked by  $H_2$  receptor antagonist, metizolide (Mick, Clin Exp Hypertension 1978, 1: 251). Also in conscious goats, a high dose of dimaprit lowered blood pressure but the latency time was well above the time of action when given on the other hand.  $H_1$  agonist, triethylamine, lowered blood pressure (Zirk, Soc Neurosci 1978, 4: 104).

The role of hi tamine in the central control of blood pressure is not defined. By using microinjection of hi tamine into the hypothalamus (Brenneis, J Neurophysiol 1969, 32: 593) and the  $H_1$  receptor antagonist, mepyramine (1977, 11: 211), these results may not be the only conclusion.

As reported in the well analyzed effect of central action of hi tamine on blood pressure, the effect of hi tamine on blood pressure is mediated by the  $H_1$  receptor. The effect of hi tamine on blood pressure is mediated by the  $H_1$  receptor.

Delbro D and Lissander B (Department of Physiology, University of Göteborg, Sweden) **HYPOTHALAMIC CARDIOVASCULAR AND GASTROINTESTINAL IMPLICATIONS**

Adrenergic nerve control is partly organized at spinal level. This is clearly the case concerning the sympathetic nerves to the gastrointestinal tract where motility and secretory tone can be decreased by activation of the spinal gastrotestino-gastrotestinal (GI-GI) reflex elicited by distension of the gastrointestinal tract or nociceptive stimuli within the abdominal cavity. Experiments involving section of the spinal cord indicate that the GI-GI reflex is under net inhibitory (supraspinal) modulation whereas cardiovascular control is generally known to be under strong supraspinal influence (cf. Ferness and Costa 1974). In the present study, experiments were performed to elucidate the interrelation between cardiovascular and gastrointestinal sympathetic effect elicited from the hypothalamus and whether the GI-GI reflex can be influenced from this structure.

The hypothalamus was stereotactically explored in chloralose-anesthetized cats. The cut vagi were frequently stimulated to their control and submaximal tone in the stomach. The volume of the latter was recorded with the balloon method together with blood pressure and heart rate.

Hypothalamic depressor or bradycardic responses were parallelled by decreases in gastric volume. However, pressor responses indicating an increased adrenergic tone on the cardiovascular system in general were likewise associated with gastric contracture whereas stimulation induced tachycardia was not clearly linked to any particular type of gastric response.

Thus the adrenergic activity in the stomach and the cardiovascular system did not always change in parallel and possible explanation was that the activity of the GI-GI reflex and cardiovascular neurons in general were independently influenced from the hypothalamus. As the animals were laparotomized a high degree of activity in

the reflex could be supposed.

In acute experiments on cats with a chronic Thiry-Vell loop, the GI-GI reflex could be elicited at will without prior laparotomy. Distension of the innervated intestinal loop elicited marked and prompt increases in gastric volume. Stimulation of the hypothalamus depressed or area decreasing blood pressure and heart rate (cf. Ikon, Johansson and Öberg 1969) abolished the gastric inhibition during the GI-GI reflex but did not influence gastric tone when the reflex was absent. It is concluded that the GI-GI reflex can be inhibited from the hypothalamus and this influence may be exerted independently of changes in cardiovascular neurons in general.

#### References

- FERNESS, J. B. and M. COSTA, *Reviews of Physiology* 1974, 60, 1-61.  
FELDMAN, S. S., JOHANSSON and ÖBERG, *Acta physiologica Scandinavica* 1969, 47, 262-270.

Sundler F, Alqvist J, Håkansson R and Lönnerdal (Department of Histology and Pharmacology, University of Lund, Lund, Sweden) **ASCENDING LOCALIZATION OF PEPTIDE HORMONES WITH SPECIAL REFERENCE TO THE ABDOMINAL ENDOCRINE ORGAN**

The last decade has witnessed virtual information explosion in the field of peptide hormones and neuropeptides. This progress has resulted from the development of immunological techniques (radioimmunoassay and immunohistochemistry). Several recent studies have indicated the occurrence of peptide hormones and neuropeptides in unexpected locations. Thus, certain peptides of the endorphin/endorphin (GH) prototype (ACTH,  $\alpha$ -MSH,  $\beta$ -lipotropin and  $\beta$ -endorphin) and of the gut (gastrin, CCK, GLI and VIP) have been found to occur in the brain. Conversely, peptides first demonstrated in the brain (such as somatostatin, neurotensin and the enkephalins) occur also in peripheral organs, notably the gut and pancreas. In the following examples are given of abnormal localizations of still other hormonal and neuronal peptides.

Certain growth hormone secretagogue GH-like immunoreactivity in the enteric parietal cells and in a population of pancreatic islet cells. Other GH analogues, while being present in demonstrating pituitary GH cells, fail to demonstrate the cells in the periphery, indicating that the immunoreactive material in these latter cells is distinct from pituitary GH.

ACTH-like immunoreactivity occurs in scattered endocrine cells of the gut and pancreas of certain mammals as well as in nerve fibers of the porcine gut. Immunohistochemical analysis using several ACTH antisera directed against different regions of the ACTH molecule indicate that the immunoreactive material is distinct from pituitary ACTH (CLIP (ACTH 17-39) and  $\alpha$ -MSH (ACTH 1-13)).

Antisera against GIP (gastric inhibitory peptide) have been found to demonstrate not only endocrine cells in the duodenum-

jejunum but also numerous endocrine cells in the pancreas and large intestine. These latter cells are identical with glucagon cells and GII (glucanin) cells, respectively. Since some GIP antisera fail to demonstrate glucagon and GII cells, the immunoreactive material in these cells is probably distinct from duodenal jejunal GIP.

Certain insulin antisera are capable of reacting with the pituitary ACTH and MSH cells. The immunoreactive peptide is chemically related to but distinct from authentic insulin. Thus, the ACTH and MSH cells contain insulin-like peptide in addition to the endorphins and the polypeptides having NH<sub>2</sub>-terminal tyrosine.

Somatostatin immunoreactive cells are abundant in the pancreas and gastrointestinal tract. In addition somatostatin immunoreactivity occurs in parafollicular cells of the mammalian thyroid and in the avian thyroid. In the thyroid the somatostatin cells contain immunoreactive calcitonin as well. In addition there is a population of calcitonin cells which does not contain somatostatin. The granules of the calcitonin cells are morphologically distinct from those of the somatostatin cells.

Several of the observations above concern those components which resemble known peptide hormones without being identical. Although they are recognized by antibodies against the peptide hormones in question they do not possess the usual immunological properties of the full peptide. This may be explained in one of the following ways:

1. Immunological cross reactivity. The immunoreaction may reflect the presence of structurally related but physiologically unrelated peptides.
2. Peptide fragments. The immunoreaction may reflect the presence of fragments (bioactive?) of the peptide rather than the peptide itself.

LENNBERG, J.M. (Dept of Histology Karolinska Institute Stockholm, Sweden) **EXTRACELLULAR SUBSTANCE & VIP SOMATOSTATIN GASTRIN/CCK AND NEUROTENSIN IN PERIPHERAL NERVOUS SYSTEM**

The presence of acetylcholinesterase (AChE), substance P (SP), VIP, somatostatin (SOM), gastrin/CCK (GAS) and neurotensin (NT) like immunoreactive material in the peripheral sympathetic (S) and parasympathetic (P) nervous system of the rat was studied using immunohistochemistry. Adrenergic systems were visualized using antibodies to the catecholamine (CA) synthesizing enzymes and histochemical reactions with acetylthiocholinesterase staining (AChE). Normal rat tissues and ganglia as well as specimens from animal subjected to nerve ligation. Observation and local visualization applications were investigated. It was found that all peptides studied (EX SP VIP SOM GAS and NT) were normally transported mainly in ant rograde direction. The following general distribution pattern and neuronal projections were found: EX was observed in 1) primarily preganglionic axons leaving the CNS via 1) the vagus and pelvic nerves (ventral ventral spinal roots terminating in P5 ganglia of S & G re piratory and urogenital tracts and 2) thoracic ventral spinal roots extending into S & G sympathetic nerves terminating in S ganglia. b) in postganglionic P5 neurons in ganglia innervating lag mucous glands S & G pharyngeal and esophageal ganglia with terminals in the parathyroid; and in Auerbach's plexus of the gut innervating lag smooth muscle, particularly in sympathetic regions and blood vessels. EX was present in 1) sensory neurons of S & G nodose and dorsal ganglia projecting with peripheral branch into S & G vagus sympathetic pelvis and also serve axons to various areas including respiratory tract heart gut S and P5 ganglia urogenital tract and skin (innervating ganglion cells and blood vessels as well as lag free nerve endings, and 2) in postganglionic P5 neurons of the gut innervating smooth muscle. VIP was observed in 1) postganglionic P5 neurons of the respiratory and urogenital tract heart (few) parasympathetic gut (both in rat and in Auerbach's plexus) sympathetic celiac submandibular and pelvic ganglia innervating 1) blood vessels and smooth muscle of the gut respiratory and urogenital tract particularly smooth muscle of sphincter regions 2) esophageal glands S & G pylorus and cervix uteri and 3) esophageal glands S & G parasympathetic nodose, esophageal, bronchial and adrenal medullary glands. Nerve stained for VIP and AChE were often closely overlapping in esophageal glands of S & G nodose. AChE-rich and VIP positive cells of S & G sympathetic and pelvic ganglia were often identical suggesting coexistence of VIP and acetylcholinesterase. b) postganglionic S neurons (mainly cell bodies and LY 51 ganglia) projecting via axillary nerve to sweat glands and some skeletal muscle rest tissue vessels. AChE rich and VIP cells were closely overlapping also in these ganglia indicating further presence of VIP in cholinergic neurons. c) some VIP got neurons project back to prevertebral ganglia (S & G coeliac and mesenteric ganglia). Neurotensin nerve axons however do not seem to be AChE-rich and d) sensory neurons of nodose and dorsal ganglia with unknown destination. However some free nerve endings containing VIP have been observed in the urogenital tract as well as sensory nerve endings in the carotid body. EX was present in 1) sensory neurons of the nodose and spinal ganglia the latter possibly projecting to S ganglia. b) postganglionic P5 neurons of the gut (mainly in Meissner's plexus of intestine) extending into the villi. GAS was found in 1) sensory neurons of the nodose and spinal ganglia. Peripheral axons in S & G pelvis and celiac nerves and partly around blood vessels and partly as free nerve endings. b) postganglionic gut neurons (mainly distal intestine) and c) neurons projecting from gut to prevertebral ganglia (see VIP). EX was mainly observed as postganglionic axons projecting via S ganglia (thoracic ventral spinal roots) into S & G sympathetic nerves terminating in various S ganglia. CA containing chromaffin cells of the adrenal body contained EX; SIF-cells EXK and SOM; adrenal medullary cells EXK and EX respectively.

Fahnenkrog, J. (Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark) **VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) A PUTATIVE NEUROTRANSMITTER**

Vasoactive intestinal polypeptide (VIP) is a highly basic octacosapeptide originally isolated from porcine small intestine in 1970. VIP has wide range of biological effects among these vasodilation, relaxation of non-vascular smooth muscles and stimulation of pancreatic bicarbonate secretion.

VIP was originally considered to be a gut hormone but recent radioimmunochemical and immunohistochemical studies have revealed that the peptide has wide-spread distribution in the body localized in neurons. In the central nervous system VIPergic nerve terminal and cell bodies are primarily located to the cortex hippocampus hypothalamus and amygdala complex. In the digestive and genitourinary tracts VIPergic nerve fibres of beaded appearance innervate small blood vessels smooth muscle and epithelial cells.

VIP is localized in vesicles within the nerve terminal from which it can be released by depolarizing stimuli. Whilst the physiological role of VIP in the central nervous system is still unknown, evidence has been provided that VIP is the neurotransmitter of the following gastrointestinal and pancreatic functions known to be mediated by non-cholinergic non-adrenergic autonomic nerves: 1) the gastric receptive relaxation, 2) the intestinal hyperemia induced by mechanical stimulation, 3) the vasodilation of the colon provoked by activation of the pelvic nerves; 4) the vagally induced pancreatic bicarbonate secretion.

submandibular and pelvic ganglia innervating 1) blood vessels and smooth muscle of the gut respiratory and urogenital tract particularly smooth muscle of sphincter regions 2) esophageal glands S & G pylorus and cervix uteri and 3) esophageal glands S & G parasympathetic nodose, esophageal, bronchial and adrenal medullary glands. Nerve stained for VIP and AChE were often closely overlapping in esophageal glands of S & G nodose. AChE-rich and VIP positive cells of S & G sympathetic and pelvic ganglia were often identical suggesting coexistence of VIP and acetylcholinesterase. b) postganglionic S neurons (mainly cell bodies and LY 51 ganglia) projecting via axillary nerve to sweat glands and some skeletal muscle rest tissue vessels. AChE rich and VIP cells were closely overlapping also in these ganglia indicating further presence of VIP in cholinergic neurons. c) some VIP got neurons project back to prevertebral ganglia (S & G coeliac and mesenteric ganglia). Neurotensin nerve axons however do not seem to be AChE-rich and d) sensory neurons of nodose and dorsal ganglia with unknown destination. However some free nerve endings containing VIP have been observed in the urogenital tract as well as sensory nerve endings in the carotid body. EX was present in 1) sensory neurons of the nodose and spinal ganglia the latter possibly projecting to S ganglia. b) postganglionic P5 neurons of the gut (mainly in Meissner's plexus of intestine) extending into the villi. GAS was found in 1) sensory neurons of the nodose and spinal ganglia. Peripheral axons in S & G pelvis and celiac nerves and partly around blood vessels and partly as free nerve endings. b) postganglionic gut neurons (mainly distal intestine) and c) neurons projecting from gut to prevertebral ganglia (see VIP). EX was mainly observed as postganglionic axons projecting via S ganglia (thoracic ventral spinal roots) into S & G sympathetic nerves terminating in various S ganglia. CA containing chromaffin cells of the adrenal body contained EX; SIF-cells EXK and SOM; adrenal medullary cells EXK and EX respectively.

Acknowledgements: Some of the studies were performed in collaboration with Drs U. Englund, J. J. Molet, M. Jodal, O. Lundgren, L. Olbe and O. B. Schaffalitsky de Muckadell.

Uvela-Hallén, E., Efendi, S., Jörhag, J. and Lundberg, J.M. (Department of Pharmacology and Physiology, Karolinska Institute and Department of Endocrinology, Karolinska Hospital, Stockholm) and Department of Surgery, University Hospital, Lund. RELEASE OF PEPTIDES FROM ENDOCRINE CELLS AND NERVES.

The aim of the study was to study mechanisms which control the release of peptides from endocrine cells of the gastrointestinal tract and from peripheral nerves. The release of gastrin as intestinal hormones was studied in acute experiments in cats. The portal vein was cannulated and portal blood concentrations of gastrin, glucagon, insulin and somatostatin were measured. The cats were provided with acute oral pouches which were perfused with 0.1 M HCl or 0.15 M HCl. Since gastric and intestinal hormones are released both into the local and into the general circulation, the release of gastrin and somatostatin level of the perfusate was also determined. Electrical vagal stimulation increased the release of gastrin and somatostatin into the perfusate and increased the release of gastrin into the portal vein whereas the release of somatostatin into the portal vein whereas portal somatostatin level decreased. The intraluminal secretion of somatostatin was enhanced only when vagal stimulation occurred at acid intraluminal pH whereas gastrin release was considerably higher at neutral than at low pH if the perfusate was fully active ion of the sympathetic nervous system or infusion of adrenaline caused a high increase in local gastrin level whereas release of gastrin at neutral pH was released, acid pH.

The peptides of the endocrine cells of the stomach are not probably released not only into the blood stream but also into the interstitial fluid surrounding the cells. Therefore, the local concentration of gastrin in the stomach is high around the cells. Since vagally induced gastrin release can be blocked by 1-fusion of somatostatin (0.5 µg/kg/h) we suggest that the inhibitory effect on gastrin release

observed at low intraluminal pH is mediated by direct effect of somatostatin on the D-cell.

Vagally induced gastrin release was reduced to 30% of control level when simultaneous activation of the sympathetic nervous system was performed or by infusion of NA (5 µg/kg/h). The inhibitory mechanism was completely independent of intraluminal pH. We think that the inhibition is caused by an adrenergic inhibition of the vagal impulses which is mediated by the ganglionic level.

Tibutamides or gibberellins given orally or intraperitoneally caused an increase of gastrin and somatostatin level in the lumen and release of gastrin into the blood stream. Obviously the secretion of gastrin into the lumen can be mediated by secretory agents applied into the local or the lumen.

Due to the unique morphological properties of the endocrine cells of the gastrointestinal tract, the rate of gastrin release is controlled under multifactorial control. Thus, the autonomic nervous system can be controlled by activation of the sympathetic nervous system but also by local factors in the stomach such as low intraluminal pH or other more different chemical compounds can influence the rate of gastrin release both into the lumen and into the local circulation.

When the vagal or brachial nerves of extrinsic origin were cut, the release of gastrin into the lumen was reduced to 10% of control level. Locally given 17 and an insulin-like substance appeared in the perfusate. Still, the intraluminal drugs, tibutamides and gibberellins added, the perfusate caused an increase of gastrin and the insulin-like substance in the perfusate.

At present, it can not be definitely stated whether these peptides are released from nerve endings or from some other source. However, if the gastrin and the insulin-like substance are released from nerve fibers, striking similarities exist between the ways by which peptides can be released from endocrine cells and nerve.

Efendi, S. (Department of Endocrinology, Karolinska Hospital, Stockholm, Sweden) SOMATOSTATIN: A HORMONE OF THE PANCREATIC B-CELLS.

Isolation and synthesis of somatostatin was soon followed by the finding that the peptide inhibits the secretion of somatostatin of peripheral hormones. Shortly afterwards we demonstrated that somatostatin is also present outside the hypothalamus in other parts of the nervous system as well as in several peripheral endocrine cells like the D-cell of the pancreatic islets.

We have investigated the possible physiological significance of somatostatin for the regulation of the functions of the endocrine pancreas by two approaches: firstly by studying the effect of exogenous somatostatin on an secondly, by trying to elucidate mechanisms controlling the release of the peptide.

Exogenous somatostatin decreases basal and stimulated insulin levels in vivo and suppresses the insulinogenic effect of glucose, arginine, albutamide and glucagon. Somatostatin inhibits basal and stimulated glucagon release.

Somatostatin release as studied in the perfused rat pancreas as well as in rats, cats and dogs. The peptide, measured by RIA using an antibody to glucose, arginine and sulphonylureas significantly stimulated somatostatin release. Intracal stimulation of the vagal nerve resulted in a decrease in portal vein somatostatin. The and their results suggest that somatostatin may play an important role as a local paracrine regulator of the release of insulin and glucagon. As for the mode of action of somatostatin, the data suggest that it is effective with

the cellular level of cyclic nucleotide may be of significance.

The clinical use of somatostatin has been explored especially in diseases characterized by hypersecretion of hormones known to be inhibited by somatostatin. However, the wide distribution and multipotent inhibitory action of the peptide calls for cautious analogues to somatostatin with specific sites have already been produced and await clinical evaluation.



H1 p. 8 (Dep. med. t. f. Physiol. gy. U. I.  
 f. k. d. Ku. pl. T. hu. and K. pl. Vi. l. d.)  
**BIOLOGICAL SIGNIFICANCE OF EXTRACELLULAR DRUG  
 BIOTRANSFORMATION OPENING REMARKS**  
 Th. li. h. g. lly. b. huo. l. dg. d.  
 b. j. it. f. h. drug. h. li. K. tly.  
 i. l. g. i. h. b. p. id. th. bi.  
 f. rae. i. ci. l. h. p. i.  
 i. lly. dnl. i. d. drug. d. th. bi. ti.  
 ne. i. h. 7h. body. h. l. f. on. these.  
 oupe. d. by. t. rui. g. hom. i. mch. i.  
 t. bollt. d. by. i. h. h.  
 Th. i. i. fi. di. g. i. bli. bi. g. h.  
 i. t. f. t. b. p. i. d. g. m. bellow. h.  
 h. h. d. i. f. b. g. i. id. i. l. K.  
 A. Had. K. p. V. 22. 239. 1955. Sem. H. p. Pari.  
 52. l. 1956. La. b. i. l. me. h.  
 f. rae. d. t. i. b. ly. ll. y. p. f.  
 d. h. b. li. i. g. i. t. b. i. t. tinal.  
 mi. fi. y. ev. p. f. r. h. i. b.  
 li. i. bi. p. (Hu. i. l. K. Physiol. l.  
 h. 53. 496. 1973). Whil. h. lyti. l. i. bod.  
 h. p. g. d. rae. ou. y. p. i. l. i.  
 h. b. f. d. id. d. j. g. f. ig.  
 oup. d. (V. i. l. K. d. Hi. E. I. C.  
 p. i. Drug. h. bellow. Kd. B. T. d. p.  
 J. na. Me. l. D. kh. l. p. 1979). It.  
 i. gi. lly. height. h. drug. me. b. li. i. g. on. yne.  
 y. em. ill. ly. d. ify. nobi. l. i. t. ly.  
 i. l. i. h. h. me. f. d. h. pi.  
 h. h. sh. xvi. h. i. nobi. p. bi. t. h.  
 b. dy. l. d. h. y. ill. p. bi. t.  
 p. d. readi. d. id. ff. by. f. r. h. h.  
 u. i. readi. bi. d. vi. h. me. onol.  
 Th. ti. en. (g. i. d. l. a) bi. b.

the. po. f. ry. f. nobi. i. hav. f. en.  
 qui. high. bi. t. f. rna. ti. i. y. lth.  
 i. d. d. by. l. d. g. m. c. bi. i.  
 d. h. ly. Th. h. p. i. drug. h. bel.  
 i. ing. ti. i. y. i. highly. iti. the.  
 i. huo. l. t. l. Th. i. t. l. i. i. y.  
 h. modifi. d. by. h. i. ti. sal. f. (Nir-  
 ou. X. d. Lo. g. H. i. Conf. g. tion. Sweet.  
 i. H. g. H. i. f. r. m. i. Xd. A. Ai. i. El. a-  
 i. /H. h. Hall. d. p. 199. 1978). Th. p. y.  
 i. f. feed. y. i. trad. prod. i. g.  
 yf. id. i. d. i. i. h. i. t. ci. l. drug. me.  
 b. li. m. in. th. way. tha. h. bl. ed. ou. tr-  
 ti. f. p. lly. dnl. i. t. d. drug. h. be.  
 h. d. (C. r. A. H. i. f. d. f. oc. 26.  
 1647. 1977).  
 p. lly. the. l. f. an. h. y. i. drug. me.  
 b. li. m. h. be. l. d. f. on. h. p. el. t.  
 f. ph. rae. ki. ti. and. l. h. ig. i. fi. en.  
 f. ext. h. p. i. ti. h. h. h. al. d. i.  
 h. v. m. Hove. d. ing. h. onp. pa. i.  
 dnl. i. tion. h. drug. h. luey. pa. i. h.  
 hi. lung. h. i. inal. we. whi. h.  
 ti. i. gh. i. t. p. i. l. i. pa. i. h. fi. h.  
 p. ff. bof. h. drug. t. lly.  
 p. l. y. y. tou. e. pa. h. li. (Sa. dy. K. h.  
 ad. Gill. J. h. J. Ph. rae. ki. bi. ph. rna. cou.  
 6. 355. 1976). i. th. ind. d.  
 h. p. i. drug. bellow. may. l. y. i. l. i. a.  
 drug. ion. T. h. i. ti. l. wall. heni. m. d.  
 y. ba. t. boll. d. thei. j. g. t. ad. ex.  
 d. h. h. h. i. l. l. m. vi. h. u.  
 ev. i. i. l. i. g. h. y. em. i. r. l. i. (J. ting. B.  
 i. l. i. l. i. g. h. Ph. rae. i. 25. 613. 1974). H.  
 i. t. l. Ab. t. H. i. f. Ceng. f. i. l. i. chow.  
 i. p. 1979).  
 i. h. t. h. i. g. i. l. h. by. th. the. ve.  
 pp. t. d. by. h. H. W. C. c. (201. 28. 0168). ad. J.  
 V. i. l. y. d. i.

Orenius, S., Graf tröm, B., Ormstad, K., and  
 Møllgård, P. (Department of General Medicine  
 Karolinska Institutet, S 104 01 Stockholm  
 Sweden) ISOLATED CELLS FROM EXTRACELLULAR  
 DRUGS AS A MODEL TO STUDY DRUG BIOTRANSFOR-  
 MATION

Isolated isolated cell from rat kidney and  
 small intestine were prepared by enzyme diges-  
 tion kidney cell using recirculating perfu-  
 sion system containing collagenase intestinal  
 cells by incubation of the intestinal mucosa  
 with collagenase and hyaluronidase.

After cell preparation 85-95% of the cells  
 were viable as judged by trypan blue and GUM  
 exclusion. No loss of viability was observed  
 within 1-3 hours.

Both cell types catalyzed cytochrome P-450  
 dependent oxidation reactions as well as conju-  
 gation with glucuronic acid sulphate and glu-  
 thione. These reactions proceeded at maximal  
 rates indicating sufficient endogenous produc-  
 tion of cofactors. Induction of cytochrome  
 P-450 oxidation by 3-methylcholanthrene was ob-  
 served using paracetamol, harmaline and benz (a)  
 pyrene as substrates. The effect was most pro-  
 nounced for benz (a) pyrene oxidation in the in-  
 testinal cell where a 10-fold stimulation was  
 achieved.

Of glucuronidation and sulphatation glucu-  
 ronidation had highest activity in both the  
 kidney and the intestinal cell. In kidney cells  
 sulphatation was about half of glucuronidation  
 whereas only trace amounts of sulphate conju-  
 gate were found in the intestinal cell. The  
 activity of these two conjugation reactions as  
 well as that of the cytochrome P-450 dependent  
 oxidation was considerably less than in isolated  
 hepatocytes. This was true also for glutathione

thione conjugate formation. However the con-  
 jugation of thiopyrene to the cysteine and  
 N-acetylcysteine conjugate reactions absent  
 in isolated hepatocytes was present in both  
 kidney and intestinal cells. Of the two cell  
 types kidney cell had the highest activity.



Vainio, M. (Department of Industrial Hygiene and Toxicology, Institute of Occupational Health, Kaarttaninkatu 1, M-10120 29 Finland) **EXTRASECRETORY BIOTRANSFORMATION IN DRUG INDUCED TOXICITY**

The enigmas of how inert chemicals can exert various adverse biological effects including genetic and toxic damage and carcinogenesis is has been much debated. It has been learned that such compound are metabolically converted to chemically active species. Thus the localisation of enzymes which occur in these active metabolites play an important role in initiating the toxicity development. For many of the chemicals which require metabolic activation to exert their toxicity (e.g. carcinogenicity) the site of action appears to be determined primarily by the final concentrations of effective intermediates available for reaction with all the macromolecules which results in a critical balance between activation and detoxication of the chemical in vivo.

Since the interaction of environmental toxins, carcinogens or mutagens with the gonads may alter the reproductive capability by effects on the gonadal germinal cells by effects on the germ cell. The results of such interactions may be impaired fertility, gonadal tumor or germ cell mutations.

Degeneration of nervous system in mammals in long-term exposure to neurotoxic chemicals appears to be at least partly mediated by the formation of reactive intermediates. The brain tissue contains enzymes which are known to be able to catalyze the metabolic activation reactions and which can explain the appearance of metabolite neurotoxicity of number of solvents (e.g. *p*-benzene disulfide).

Guenther, Thomas W., Mena, Raymond W. and Herbert, Daniel W. (National Institute of Child Health and Human Development and National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA) **STUDIES ON THE CONTROL OF ARYL HYDROCARBON HYDROXYLASE IN RAT ADRENALS: INDUCTION BY ACTH BUT NOT BY POLYCYCLIC HYDROCARBONS**

Aryl hydrocarbon hydroxylase (AHH) activity in the rat adrenal cortex is specifically located in the microsomal fraction 95 percent of the total AHH activity; microsomal while only 10 percent of the total P-450 content; microsomal. This AHH activity can be distinguished from the major P-450-dependent adrenal microsomal steroid hydroxylase, progesterone 21-hydroxylase, since the latter is strongly inhibited by naphthoflavone but the former is not. Hypophysectomy decreases adrenal microsomal AHH activity to 6 percent of its former level; progesterone 21-hydroxylase level decreases to 38 percent of control values. An electrophoretic band at 57,000 daltons which migrates identically to the band corresponding to AHH activity from 15 microsome preparations is also markedly reduced by hypophysectomy. Exogenous ACTH treatment restores both AHH activity and the electrophoretic band to normal level. However, AHH activity is not induced either in the intact hypophysectomized rat by high doses of 3-methylcholanthrene or 2,3,7,8-tetrachloro-g-dibenzodioxin.

These data show that AHH and progesterone 21-hydroxylase are different enzyme activities and are likely associated with different microsomal AHH has properties very similar to liver microsomal AHH (i.e. sensitivity to naphthoflavone inhibition and is presumed as-

Recent evidence also suggests that environmental mutagen and their metabolism within smooth muscle cells in the rat may be involved in the potential to initiate the genesis of peritoneal diseases. The biotransformation of promutagens into active metabolites would then initiate the formation of monoclonal thymic lymphoma plaques.

Lenticular opacification or cataract may be induced also by chemicals and drugs. Naphthalene and paracetamol react intermediates are bound covalently in the lens of mice producing a reversible opacity in the lens. These ophthalmological findings may be important also clinically with regard to occupational exposure to organic solvents. Among other parameters changes (nuclear cataract, cortical and subcortical opacities) have occurred with increased frequency as compared to silvery eelings.

Only few examples have been given here to indicate the significance of the extrahepatic biotransformation in drug-induced toxicity. Although quite large perspectives exist, it is still too early to predict its ultimate importance.

association with the 57,000 dalton electrophoretic band) but their sensitivities to inducers are distinctly different. That adrenal microsomal AHH is inducible by ACTH and not by polycyclic hydrocarbons suggests that the mechanism of induction of this enzyme is quite different from that postulated for liver AHH even though the enzymes themselves are quite similar.

Owing, E. B. (Institute of Zoophysiology University of Oslo Norway) TRANSDUCTION MECHANISMS IN THE VERTEBRATE OLFACTORY RECEPTORS.

The mammalian olfactory receptors have long cilium-like protrusions that are embedded in the mucus of the sensory epithelium. Each cell has about 17 cilia. The inner filaments have the usual 9+2 structure but lack dynein arms. Presence of cilia on the olfactory receptors of amphibians has been shown to be essential for the development of the electro-olfactogram, EOG. Some species of lower vertebrates have two types of receptors with cilia or with microvilli. The membrane of the receptor cilia bears intramembranous particulate IPFs at density 19 times higher than that of the neighbouring cilia of the respiratory epithelium. These particles have been suggested to contain the receptor structures recognizing the odors. In frog there is a positive correlation between the density of IPFs and the amplitude of the EOG to standardized olfactory stimuli. From the dog olfactory epithelium, protein fraction with specific binding to androstenes has been extracted by affinity chromatography. Antibodies to this protein fraction applied topically to the olfactory epithelium of mice decreases the EOGs to androstenes and other odors.

Recording of the electrical activity of the frog receptors show that most cells have low spontaneous activity. Most of the stimuli that influence the activity of the cell tend to increase the spike discharge only. Few will suppress the discharge. Increasing the concentration increases the probability that the cell will respond to particular stimulus. Only tenfold increase in concentration is needed to reach various discharge frequency. A single receptor can respond to one or several groups of odors. The evidence for the spatial concept of quality discrimination in the olfactory system will be discussed.

Järnblantz, I. (Department of Psychology University of Helsinki, Finland) PROPERTIES OF MECHANORECEPTIVE FIBERS SUPPLYING THE HADRY SKIN OF THE HUMAN HAND.

The method of microelectrode recording from the human peripheral nerves (Wallin, A. and Hagbarth, K.-E., Exp. Neurol. 59: 31, 1976) enables detailed analysis of the types and properties of single peripheral nerve fibers in man. At the present the main knowledge on the branching properties of the mechanoreceptors is based on electrophysiological work in different animal species. The present paper describes properties of mechanoreceptive fibers supplying the hairy skin of the back of the human hand.

The fiber sample consists of 250 single fibers in the superficial radial nerve. On the basis of the response to static pressure 63 fibers could be classified as rapidly adapting (RA) fibers and 176 as slowly adapting (SA) fibers. On the basis of receptive field characteristics and responses to stroking the skin 33 SAI and 69 SAII fibers were identified.

The receptive fields of the fibers ranged from optically field (below 1 cm in diameter) to large fields of several cm. Some fibers responded exclusively to stimulation over the receptive field. Conduction velocities of the fibers ranged from 3 to 90 m/s, the mean being 54.7 m/s. There were no significant differences between the conduction velocities of the different fiber groups.

The static discharge of SA fibers was studied by interval analysis during continuous pressure exerted by Frey hairs or by the tip of an electromechanical vibrator (intensity of 1 mm). The static discharges of SAI fibers are small, quite variable, the standard deviation of the interval histogram being in most cases over 50% of the mean interval (coefficient of variation, CV). In contrast, most SAII fibers had an extremely regular static discharge (CV below 10%). 80 SAII fibers had resting discharges of 3.8 ± 0.8

imp/s.

The dynamic discharge of the fibers was studied when using tactile pulses applied to the receptive field by the tip of the vibrator. Pulse amplitude was varied between 50-850  $\mu$ m and pulse frequency was 20, 60, or 120 Hz (one spike sine-wave). The latency of the first impulses in the response was dependent on the stimulus amplitude, being reduced by 5 ms from the lowest to the highest amplitude on the average.

Stimulus-response functions of the fibers were calculated when using three different response measures: 1) the number of impulses, 2) the first interval, and 3) the mean interval. The fibers responded to the pulse indentations with few impulses initially with 7. It was typical of the fibers that they usually had an optimal pulse frequency at which they elicited the maximal number of impulses. When the number of impulses was used as response measure the stimulus-response relation could in most cases be described by power functions with exponents below 1.00 over the whole frequency range. The first interval or the mean interval in the responses did not have an exponential relation to the stimulus amplitude. The data indicate that the amplitude of pulse indentations is coded by the number of impulses both in SA and SAI fibers, whereas coding of the pulse velocity is based on the differential sensitivity of the fibers to pulses of varying velocity.

**Band, O** (Dept Physiol, Veterinary College of Norway, Oslo, Norway) **THE ROLE OF  $Ca^{2+}$  IN THE HAIR CELL TRANSDUCTION PROCESS**

Recent intracellular recordings from hair cells in a wide range of acoustico-lateral systems show receptor potential  $i$  responses to mechanical stimuli. In the mammalian cochlea and vestibular system the hair cell face as endolymph with potential exceeding +120 mV and where  $K^+$  (150 mM) is the dominant cation. The driving force for  $K^+$  is inward through the pax and outward through the cell body suggesting that  $K^+$  might carry the receptor current. Cupulae of the amphibian lateral line are exposed to fresh water where the dominant cation is  $Ca^{2+}$ . The mechanosensitivity of the organ depends upon the  $Ca^{2+}$  concentration in the medium and is blocked by  $Ca^{2+}$  competitors (Band, J. comp. Physiol. 1975 102 27) indicating that  $Ca^{2+}$  carries the receptor current. However, ion selective electrodes revealed  $K^+$  concentrations up to 180 mM and corresponding potential up to 50 mV in lateral line cupulae (Russell & Sellick, J. Physiol. 1976 257 245). An electrogenic  $K^+$  pump was postulated and it was suggested that  $K^+$  carries the receptor current. In high  $Ca^{2+}$  low  $K^+$  solution amphibian hair cells are electrically excitable (Kodaspath & Corey, Proc. natn. Acad. Sci. USA 1977 74 2407). The electroreceptor cells in the organ of Lorenz  $i$  which are embryologically related to hair cells and possess  $Ca^{2+}$  rich canal fluid similar to  $Ca^{2+}$  spikes in the presence of TEA (Clusin & Bennett, Biol. Bull. 1977 151 429).  $Ca^{2+}$  passively distributed in amphibian lateral line cupulae which leads to low  $Ca^{2+}$  concentrations of  $10^{-3}$ – $10^{-4}$  M due to the positive endocellular potential (McDonnell, J. exp. Biol. 1979, in press). The inward

driving force for  $Ca^{2+}$  is not increased by the electrogenic  $K^+$  pump, hence the positive endocellular potential tends to exclude  $Ca^{2+}$  from the cupula. It is therefore likely that  $K^+$  is the main carrier of receptor current in these organs even if the low intracellular concentration of  $Ca^{2+}$  still causes an inward driving force for this ion. Similar arguments are applicable for the cochlea where the  $Ca^{2+}$  concentration in the endolymph is  $3 \times 10^{-3}$  M (Søndergaard & Warren, Nature 1978 271 377). The endolymphatic equilibrium ion concentration relative to the perilymph is  $7 \times 10^{-3}$  M indicating that  $Ca^{2+}$  is actively transported. It is possible that  $Ca^{2+}$  controls the membrane permeability for  $K^+$  (Neech & Standen, J. Physiol. 1975 249 211). Clusin & McDonnell (1975 256 425) demonstrated that dihydropyridine from polyphosphoinositides in inner ear tissue (Schacht, J. Acoust. Soc. Am. 1976 59 940). These lipids are believed to act in the control of membrane permeability. Flock et al. (Acta Otolaryngol. 1977 83 85) have recently shown that the hair cell cilia act as stiff rods pivoting around their base where maximal membrane distortion will occur. The stereocilia contain highly organized actin filaments. A similar actin pattern is seen in the sperm acrosomal process. Formation of this stiff protrusion starts when the sperm contacts the  $Ca^{2+}$  rich egg jelly. It is tempting to speculate that  $Ca^{2+}$  influences the mechanical properties of the sensory hairs and thus the coupling between the transducer membrane and the mechanical stimulus transduction. The mechanism of hair cell transduction is unclear.  $Ca^{2+}$  seems to have a complex role possibly acting at several different levels.

**Smith, S. and Mante, J.** (Department of Zoology, University of New South Wales, Kensington, New South Wales, Australia) **LOW-INTENSITY SPREAD OF ADAPTATION IN THE VISUAL SYSTEM**

Photolysis of rhodopsin in the di membrane of the rod  $i$  that process which decreases the amount of signal carrier molecules (Ca<sup>2+</sup> QMP) in the cytoplasm. This change in concentration of the signal carrier is causing a loss of sodium channel leading to hyperpolarization of the cell. The delayed time course of photoreceptor responses is related to the slow release of the signal carrier (retention) before the channel is closed. Both background light and an exposure bleaching of the rod  $i$  rhodopsin bring about a displacement of the stimulus-response curve predominantly in the direction of large stimulus flash intensities. Two different types of mechanisms causing this displacement may be suggested: 1) a local adaptation meaning that the photoreceptor brought about by the adapting light affects only their own displacement by decreasing the release of the signal carrier and 2) spreading adaptation meaning that the displacement proceeds from the bleached diolward possibly in the whole rod. For example, the photoreceptor could directly affect the amplification mechanisms in the cytoplasm or they could produce a secondary displacement which diffuses to other diol membranes.

We have tested the spreading adaptation by comparing the effect of two kinds of bleaching and stimulating light. Firstly, light which is preferentially absorbed by the diol end of the rod and secondly, light the absorption of which is nearly homogeneous distributed in the rod. The inhomogeneous

bleaching was achieved by using an oblique blue-green light beam hitting the receptor of the flat-mounted retina at an angle of  $10^\circ$ . To further our diol absorption this light was polarized with a least  $i$  vector in the plane of the diol membranes. Thus 70% of the incident light was absorbed in the diol one-third of the rods.

Blanching several percent of rhodopsin in an isolated frog retina lead to a larger transduction of the sensitivity (termed as adaptation) and to a small permanent decrease of sensitivity. The permanent displacement of the stimulus-response curve to the right (log-log plot) after 10% bleaches were as follows (log unit):

Stimulus	Inhomog	Homog
Inhomog bl	0.79	0.54
Homog bl	0.52	0.21

If the light adaptation were local the inhomogeneous bleaching would desensitize only the diol end of the rod and thus the displacement measured with homogeneous light should be less than after an equal homogeneous bleaching. This is clearly not the case, indicating that the process causing permanent change spread in the rod. A further unexpected observation is that blanching 10% preferentially in the diol tips has a much larger sensitivity-reducing effect than blanching 10% homogeneous in the rod (observe that this is true when the stimulus is homogeneous).

The transient (incomplete) recovery of sensitivity during 30 min after the bleaching was nearly equal in magnitude when measured with homogeneous inhomogeneous light. Thus the process causing transient adaptation also spreads within the rod.

33

K1 sling, K. M (Dependent of animal nutrition on the seed in University of Agriculture Sciences Sweden) EFFECTS OF ETHANOL CONSUMPTION ON MITOCHONDRIAL STRUCTURE AND FUNCTION

The rat  $\beta$  thalassaemic bulimic 1 th 11  
might be affected by the eat ty  $\beta$  en yee  
idi i g thalassaemic eat ity  $\beta$  system  
transp to g reducing equ alent i t the  
mitochond i and th ability  $\beta$  its had  
to ii these reducing equale t

Th t take reducing equivalent  
 Th t bolise f thenol i t f re ith  
 d t itochondrial function f the  
 d t f tly so d d the ac ty f the  
 t said yel The ffect probably  
 raised by the l redc tat produ d by the  
 nal oxidation I ontra t t to te thenol  
 into oxidation hron thenol onsumpt on  
 acc ted th ultra trustu l onang in th  
 tochond i and inc d mit hond al fre  
 gility f th more hro thenol ing tion  
 also cause t ration f tochond l tru  
 t re nd funct on i ti su wh re thenol  
 not d ed g brain skit i ti su l end  
 pence Th the hron onsumpt f  
 thenol appe re t produ p tent hang  
 tochond independent f th ac  
 ffect p produ d y the t bolise f thenol  
 Th l tochond l th jo  
 p t f the ac tal shyde f read f on thenol  
 The ac t idenhyde lav l the blood unmore  
 and ac tal shyde ha been shown int f re  
 th mitochond al f net on ou  
 ti su

34

Niilbom, M. (Research Laboratory of the  
Finnish Alcohol Monopoly (Alko) and Department of  
Neurology University of Helsinki Finland)  
TOXICOLOGICAL AND NEUROLOGICAL PROBLEMS OF  
ACUTE ETHANOL INTOXICATION

The lethal dose of ethanol is not fixed  
 value. It occasionally there are sports f  
 human fatalities recorded by relatively low  
 ethanol levels and conversely, if individual  
 surviving considerably high ethanol concentrations.  
 Autopsy findings suggest that thyroid rate may  
 influence the acute toxicity of ethanol bu  
 otherwise there is little evidence to be  
 the factors responsible for varying the lethal dose  
 in humans.

```

Thomson
    mortali y f ts t thanol colesomy i
modified by ge thyroid tat and previus
thanol exposure Young ts tol high
dose f ethanol then id comm perhaps becase
the h other total body wate content cause
these dose to result in lowe concentr tione
f thanol i the body wat i hyperthyroidism
the t f thanol equilib tione i increased
whil i hypothyroidism i is decreased Thi
may t i as partly explain why the l thal dose
f thanol i differen f hypo- hype and
ethyroid ts

```

[illegible]

Recent investigations indicate that ethanol derived acetaldehyde is usually not transported to the liver, if formed there. Accordingly, acetaldehyde may contribute only lightly if at all to the cerebral effects of acute ethanol intoxication.

Some epidemiological data suggest that the occasional thrombotic infarction seems to carry an increased risk of ischemic brain infarction. Although this does not imply a causal relationship, it is tempting to speculate that effects of thrombotic or hemocoagulation and cerebral infarction are involved. The thrombotic-induced high coagulability of blood may lead to an increase in thrombosis. This effect on the other hand is known to lower down cerebral blood flow. In addition, it is well established that platelets and cells have been observed in the conjunctiva of both experimental animals and man, due to acute, but also chronic, inflammation.

One may ask does tharol induce lodging of blood hast be fatal outcome in tharol poisoning? Does plugging f capill is by glyx ted red cell induce cereb l anoxi during i toxi tion? If so doe this effect contribs the occurrence f cereb l trophy and deessit in alcoholics? Does tharol influence the cell memb ases f cereb l vessel wall and thereby cause f cereb l vessel

will not necessarily prove that information?  
I order to resolve some of the above  
mentioned problems both acute and long-term  
effects of ethanol must be studied. One  
direction of research should be the  
examination of the ethanol-induced cell  
membrane which is weak in animal  
chronically fed with ethanol and the effects of  
ethanol on the function of cell  
and its contribution to toxicity.

Winkel, K. (Department of Clinical Physiology, Hvidovre Hospital, University of Copenhagen, Denmark) HEPATIC AND EXTRAHEPATIC ELIMINATION OF ETHANOL IN MAN WITH SPECIAL REFERENCE TO LIVER DISEASE

This communication surveys studies from the department (1, 2) of hepatic and extrahepatic ethanol elimination (EER) in liver disease with comparisons to normal man. Results of standard intravenous and steady state infusion technique of isolation of EER are compared. Hepatic EER is determined by hepatic venous catheterization.

The standard intravenous injection technique was estimated total body EER by 15% (p < 0.02). Single cases of EER determined in high blood concentrations (about 30-50 mmol/l) in man and isolated perfused pig liver failed to demonstrate an increased EER and the reason for the overestimation is thought to be due to uneven distribution of ethanol. This is substantiated by the findings (1) that while the blood elimination curve from normals were rectilinear, considerable curves from the patients were upwards concave, this being more pronounced when major fluid retention was present.

Contrary to several earlier reports we find (1) that EER (intravenous injection technique) on the average is diminished in liver cirrhosis (normal 1.77 mmol/min, SD 0.62, 13 cirrhotics 1.77 mmol/min, SD 0.49, 22 p < 0.001) and that the decrease seems to be correlated to the degree of liver disease. However, very considerable overlap between EER of normal and patients exists.

The hepatic venous catheterization studies (2) show that extrahepatic elimination (cont.)

(cont.) is saturated at the same concentration level that of the hepatic and that it constitutes 40% of total body EER. Apparent extrahepatic elimination as well as extrahepatic elimination by rough estimate found to be about 0.2 mmol/l.

The significance of these basic data will be discussed with reference to ethanol kinetics to the use of EER as an indicator of quantitative liver function (comparison with galactose elimination) and to the possible role of the considered extrahepatic elimination in man.

References: (1) Schlichting P, Heilund-Carlson P, Christiansen E & Winkel K. Ethanol and galactose elimination rate as measure of liver function in man. To be published. (2) Utne H E & Winkel K. Hepatic and extrahepatic elimination of ethanol in cirrhosis. To be published.

Gadeholt G. and Meland J. (Department of Pharmacology, Institute of Medical Biology, University of Tromsø, Norway) EFFECTS OF LONG-TERM ETHANOL TREATMENT ON DRUG METABOLIZING ENZYMES

The hepatic microsomal drug metabolizing enzyme (CME) as sensitive to environment and diet. Several different models have been used to study effects of chronic high-dose ethanol (CME) on CME. A high dietary intake of ethanol may lead to dietary deficiencies and CME may become depressed because they depressed by deficient diet. If CME never decreases is accompanied by increased enzyme activities compared to zero time alone. Untreated alcoholism had control ethanol in diet. In diet can hardly be questioned. No starvation effects would then be present because both groups have unrestricted access to food. If on the other hand the control group is pair-fed diet more palatable than the ethanol containing diet, the control animal will starve in the interval between feeding the daily ration and the next feeding starvation effect may then become apparent in the control group. An unbalanced control diet could also contribute to changes in the control group.

If the CME group and the pair-fed control group are compared the differences between the groups may be the result of either ethanol effect starvation or the control diet effect. If CME and control diet have opposite effects on CME the result of comparison will be large difference between the groups. It is not however possible to ascribe the entire difference between the groups to the inducing effect of ethanol. Reports of ethanol as strongly induce of microsomal drug metabolism have not taken into account the possibility of control diet effects.

Of central interest in drug metabolism are the microsomal systems consisting of cytochrome P-450 and the associated NADPH dependent reductase system. Properly designed studies indicate that ethanol induces specific cytochrome P-450 with unique properties above by cyanide titration (Czerny Gaylor 1973) inhibitor studies (Ullrich et al 1975) activity studies (Villeneuve et al 1976) and electron spectroscopy (Omishi & Lieber 1977). The P-450 isolated after CME has higher turnover numbers for ethanol (Omishi and Lieber) and aniline (Villeneuve et al) than P-450 from phenobarbital or 3-methylcholanthrene treated rats. Induction of aniline hydroxylase activity can be shown in crude microsomal preparations together with increased P-450. One can expect that CME rate will have accelerated elimination of drug whose major metabolites are formed by microsomal aniline hydroxylase activity.

NADPH-cytochrome c reductase is the most important electron donor of microsomal cytochrome P-450. Per unit body weight this activity does not increase compared to control time (in rats) after chronic ethanol treatment. The increased in vivo elimination of drug after chronic ethanol treatment then probably at least in part result from the quantitative and qualitative changes in the hepatic microsomal cytochrome P-450.

37

Has iaga I Savol ien M Lehtonen M and  
Hiltunen K (Depa ient i Medical Biochemi lry  
University f Oul Finland): EFFECTS OF ACUTE  
AND CHRONIC ETHANOL ADMINISTRATION ON HEPATIC  
LIPOGENESIS AND TRIACYLGLYCEROL METABOLISM  
MECHANISMS AND MEDIATORS OF ACTION

The mechanisms f the development f th cut  
and chroni th i indu ed f tly li i not  
known i det li t th aymal i vel Although  
etretrepati f ct may ont ibut t the de-  
velopment f ths condition most f th data  
gathered hith rt suggest f p ias ily hepati  
darengas is

The f tly li d velopi g ft ingi  
i rg dos f than li t ou t enhanc d  
lep ti f tly li d yth judged from dat  
from xpe iue t in vivo and i pe lused li  
Th i no videa f d cre d rat f  
liposret is tion ith Th gly rol 3  
ph sphat cytl snaf how q t limit d  
capa ity f long t rm gol ti and glyceral  
3 ph sphat aileibility has been gested as  
mechanism f regul tion f f tly id t ri  
fi tion

Phosphatidat ph phoby d i has th lowe t  
ct i ty f the ex yae f the t i cylyl i  
synth i i g p thway and its ct i ty changes  
i posu t i t ry met bol man pul  
ti Moreover th ct i ty f ph sph  
phosphoyd ias increases t three-f id value  
ft an cut than i loadi g (i). The mech  
ism f thi i creas remains to be t bli hed  
The effect i not direc ly linked t than i  
met b li s because th ct i ty can be in  
cre ed in sa i l ted perfus d li. However  
unde iue t d ondit ons the phosph tid t pho  
phoydrelase ct i ty hoes posit ve rel  
tion with th h p ti glyc rol 3 phosph t con  
ent tion is ting that small-mol cul

weight met bolit may be invol d in the gu-  
l t on f thi xyme (2). Cortis i induc  
ph sph tid t phosphoydrol se by ds ti cny  
i nait ve mechanism i i an i n i l ted  
perfus d li rm (3) and denc ha b en p  
ented f i nrease d plasma cort i l vel aft  
cut thanol i adi g Th dat is that  
phosphatidat phosphoydrolas may be ne f th  
i y en ymes i the thanol induced cmeti tion  
f f t in th liue

The mechanism f the developme t f the h on  
i than li f tly li y b i rti lly dif  
f t Und control d ondit ion specifi  
ff ct f than i on f tly id synthet d  
ing long t rm than i dmi t tion can be  
rul out (4). Mo sov ons f the ctyl  
CaA and NADPH-gen rat i an yae howd reas d  
ti ti ft ontinued thanol dmi t  
tion The i reas in phosph tid t phospho-  
hydrol se ft an cut thanol load lev l  
p rti lly ff duri g continued th mol dmi-  
i tration The view emerges that during ch on  
than i int h the rbohyer t ad lipid on  
text f th di s effects the hep ti lipogee-  
i ad lipid cmeti t on E t nation f the  
ont bution f the variou enzyme ti ty  
hanges during long t rm xpe iue i and  
diff cul by the rap d t iue t f t dy  
t t lipid on i tion i the li er

- 1) Sav i ien, M (1977) Bi chem Biophy R  
Commun 75 511
- 2) Savol ien M & Ha i en I (1978) Biochem  
J 176 895
- 3) Lehtonen M Savol ien M & Ha i en I  
(1979) FEBS Le t 99 162
- 4) Savol ien M, Hiltunen K & Ha i en I  
(1977) Biochem J 164 169

38

Orshk, K.L. (Department of Physiology and Medical  
Biophysics University of Uppsala Sweden) THE  
ANIMAL AS A SCIENTIFIC INSTRUMENT

All research is depending on different kind of instru-  
ments. In biology and medicine the laboratory animal is  
very important instrument both as qualit ative and in  
quantitative analysis. As for any other type of instru-  
ments this one must be sensitive have high predict ion  
and be reproducible. For several physical instruments  
like balance photometers etc ths has always been  
satisfying at best for the laboratory animal has and  
troubling not been. The sci ad has been forced to be  
content with widely scattered results. This has slowly  
been blamed biological variability but i many case  
the blame i to be put on the scientist himself.

We must remember that living being has homeo-  
static mechanism by which i trie to keep its internal  
milieu constant. We have control mechanisms for body  
temperature blood pressure blood sugar number of  
blood cells and practically all other parameters in the  
body. As soon as something intends to interfere with  
this quality the body has the ability to counteract in  
order to restore the original situation. This property  
the most remarkable difference from physical instruments.

We may call all the factors that i some way inter-  
fere with the constancy of the body parameters as  
stress factors. This is wide definition and will in-  
clude actions of microorganisms like bacteria and  
viruses and from parasites i also include the effect  
of the nutrition which constitutes heavy load on the  
body. It include all types of environmental factor not  
only physical ones like temperature humidity light and  
so forth but also social factor like number of animal  
in the cage relations between the animals and the  
caretakers. All these parameters will be important  
for the experimental result and may introduce serious  
error. A general rule must be to keep the parameters  
constant and if possible under control.

Let us take an example in homeostatic animals the  
body temperature is kept constant even if the ambient  
temperature changes. It is quite obvious that two  
genetically identical animal may keep the same body  
temperature and still be quite different because one  
animal may have to defend its temperature against cold  
while the other is sitting in comfortable environment.  
They use different amounts of their regulatory capacity  
and that makes them respond differently to the  
experimental situation. The results will be accordingly  
It is imperative that the scientists are aware of the  
above facts and it is equally important that all categories  
of people handling laboratory animal must be well  
trained. Finally the quality of the animal, their micro-  
biological state, the animal quarters and the husbandry  
must be of acceptable quality.



Carpenter, J. (Inst of Toxicology National Food Inst DK 2650 Søborg Copenhagen Denmark) THE NEED FOR BETTER SPECIFICATION OF THE ANIMAL MODEL IN THE TEST SITUATION

It is essential to realise and carefully evaluate all the factors which may affect animal studies. By ignoring the potential impact of an erroneous extrapolation incorrect conclusions may be the result. This will make the scientist vulnerable to scientific criticism and from an ethical point of view the scientist may be blamed for using animals for worthless experimentation.

In general the specified animal model offers the following advantages: Reliability, reproducibility, lower number of animals, reduced time and cost, fewer errors in extrapolation to the same or other species including man.

An important but also depressing survey has been published by Lang & Vessell (Fed Proc 1976 35 1123). They assess the description of the animal models in 4000 articles from 7 selected biological journals: 1 percent specify sex, 58 percent strain, 34 percent age, 19 percent bodyweight, 30 percent population density, 8 percent cage type and size, 7 percent sex, 10 percent bedding, 10 percent type of food, 26 percent feeding frequency, 32 percent temperature, 6 percent humidity, 12 percent ventilation, 0 percent photoperiod, 6 percent conditioning, 9 percent selection, 9 percent handling, 0 percent.

This illustrates the poor background information for animal model normally given in the literature. Also the test situation in itself calls for clarification: 1. The publications. Examples will illustrate this.

2. Daily and seasonal variations: Body temperature, blood pressure and blood constituents vary during the day. Seasonal balance and behaviour in female species during the estrous cycle. Corticosterone induced 1 ft per 1 ft mice show variation during the year. 3. All when the study was made.

Blood sampling Blood cells, enzymes and hormones may vary considerably between sampling sites and with sampling technique. 4. If where, when and how.

Urine sampling Sampling time relation to the possible dosage regimen, time interval since the previous voiding of urine or sampling should be indicated.

Anaesthesia An anaesthetized animal is a very special model. Measurement and analytical data are in many instances representative for the anaesthetized animal only. Specify anaesthesia.

The test substances should be well defined, have specific effect on stating the degree of purity and remaining material must be characterised, intermediate and impurities from the synthesis.

Pharmacological and other preparations How is the preparation made? Give name of registered formulation and identify pharmacopoeial preparations. Bioavailability is very important; constituents may bind the active compound.

Administration of compounds and preparations How and where? Intramuscular and oral concentration and volume of solutions as it has an influence on the absorption from the application or injection site. Oral inhibition or distal inclusion do not give identical results.

Postoperative care Scientist should pay more attention to this. Proper surveillance and instructions are important to ensure good health. After anaesthesia when recovery time effect may still persist the injection of antibiotics may act synergistically and even kill the animal. Describe treatment.

Euthanasia 1. Procedure and time of killing. 2. Urine sampling Describe handling of animal immediately before killing, the sampling technique and time sequence.

Apparatus/equipment Substances likely to interfere from plastic tubes, bags and catheters may be toxic and interfere with measurements. Give brand name/quality.

Conclusions These and other parameters are included in the recommendation on from the International Committee of Laboratory Animals (ICLA Bull 1978 no 42). This recommendation is an important check list for all authors as well as for the editors and reviewers from biological journals.

40  
Kjartan, K. (Laboratory Animal Centre Model School Hannover (Germany)) HANDLING AND EXPERIMENTAL PROCEDURES FREQUENTLY OVERLOOKED AS STRESSORS AND SOURCES OF TECHNICAL ERRORS IN EXPERIMENTS WITH ANIMALS

Blood, heart rate and physical activity in laboratory animals show great variation in group and individual animals. This is largely due to the individual component of stress and may be caused by 1. The response capacity of normal handling and experimental procedure. This was investigated by recording time rate of 25 endocrine free laboratory and male rats. 2. The confrontation of group and individual handling (stress).

3. The effects of procedure on physiological and transport laboratory animals. This was investigated by chemical analysis of urine (creatinine) and delay (immunological food) up to 100 days acclimatization.

4. The type of blood sampling procedure. This was compared by comparing the procedure of blood sampling on blood hormone levels and on the other hand on small molecular blood components.

The time of day and rhythm (4-6 hours) of blood sampling and the time of day of blood sampling were investigated by these results. The body temperature and the motor activity of undisturbed observed over long periods of time.

Östmark, K. (Department of Physiology and Medical Biophysics University of Uppsala Sweden) SOME ETHICAL ASPECTS ON ANIMAL EXPERIMENTATION

The important question whether or not the man has the right to use animal for his own purposes will not at all be discussed here. We may consider that questions answered by the mere fact that we are using animals in biological and medical research. However, important to remember that ethical problems or by no means solved by the latest in ethical problems. It is judgement between the severity of the experiment on the animal and the purpose of the experiment. It must be considered unethical to make an experiment on an animal if there is no sound scientific problem behind the experiment. Decision is ethical problems is judgement of the relation between severity and purpose. Up till now the scientist himself has made that decision. Probably most scientists have fulfilled that responsibility with high ethical standard. It is however always risk that paediatric or any professional gets blind for what he is doing in his daily work and therefore it would be an improvement if he would discuss his experiments with some fellow-researcher before the start of the experiment.

In Sweden ethical committees for that purpose have been established. They try to be of help to the scientists without bureaucracy. In such judgement the members of the committee must convince themselves that the project is scientifically sound and that it is not an unnecessary repetition of already made experiments.

That the problem cannot be solved without the use of animals and that the experiments cannot be modified so to involve lower degree of animal distress.

Flora E. and Sand T. (I tit t f M dical  
Biology University of Tromsø, Norway)  
CHARACTERIZATION OF THE HEART VENTRICLE BY ITS  
PVT PLANE

We have searched for load in right  
ventricle of the performance of the heart.  
The right ventricle of the heart is loaded  
by the pressure of the blood in the right  
ventricle. The pressure of the blood in the  
right ventricle is determined by the load  
of the heart. The load of the heart is  
determined by the pressure of the blood in  
the right ventricle. The pressure of the  
blood in the right ventricle is determined  
by the load of the heart.

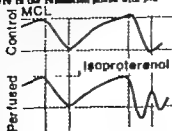
Three-dimensional diagrams of PVT and time  
after onset of action were constructed.  
The three-dimensional diagrams of PVT and time  
after onset of action were constructed.  
The three-dimensional diagrams of PVT and time  
after onset of action were constructed.  
The three-dimensional diagrams of PVT and time  
after onset of action were constructed.

For the load of the PVT plane, the  
load of the PVT plane is determined by  
the load of the PVT plane. The load of  
the PVT plane is determined by the load  
of the PVT plane.

The PVT plane is a complex parameter  
representing the load of the heart.  
The PVT plane is a complex parameter  
representing the load of the heart.

Rohrer, A., Lohr, J., and KJ P. (Institute for Experimental  
Medical Research, University of Oslo, Norway).  
ASYMPTOTIC MUSCLE FIBRE FUNCTION IN THE LEFT  
VENTRICLE DURING LOCALIZED ISOTROPIC STIMULATION  
WITH ISOPTERENOL.

The purpose of the present study was to examine myoelectric  
function in the left ventricle of anesthetized open-chest dogs  
where localized part of the muscle mass was selectively  
stimulated with the  $\beta$ -adrenergic agonist isoproterenol. A  
stimulus was established between the left aortic artery and  
the anterior descending coronary artery for infusion of isopro-  
terenol. One pair of plane-electric elements was inserted  
into the area to be stimulated (the isoproterenol and the  
control area). The distance between the two plane-electric  
elements was continuously recorded by an ultrasonic  
technique to estimate muscle fibre function. Small doses  
of isoproterenol (0.1-0.5  $\mu$ g/min) which induced no general  
hemodynamic effects, elicited the contraction pattern of  
muscle fibres both in the stimulated and in the control area.  
In the control area, muscle fibres were stretched in early  
systole due to the greater rate and magnitude of tension  
development in the stimulated than in the non-stimulated  
muscle fibres. When the control area was later stimulated  
isoproterenol-stimulated, muscle fibres were stretched  
because they already were in the relaxation phase with low  
tension. On the other hand the control area  
contracted to lower MCL. Hence administration  
of isoproterenol to part of the left  
ventricle results in synergistic contractions  
and reduces the overall  
performance so that  
stroke volume may not  
rise despite extensive  
isotropic stimulation.



Flora E. and Sand T. (I tit t f M dical  
Biology University of Tromsø, Norway)  
MATCHING BETWEEN RIGHT HEART VENTRICLE AND ITS  
ARTERIAL LOAD

The right ventricle (RV) is traditionally  
considered to be flow pump. This should mean  
that the work done is assessed directly in  
proportion to the load resistance. This  
characteristic of the RV was tested by  
isolating the heart from the pulmonary artery  
and the right ventricle (RV) and the pulmonary  
artery (PA).

RV was flow decreased with increasing  
load. The flow was decreased with increasing  
load. The flow was decreased with increasing  
load. The flow was decreased with increasing  
load. The flow was decreased with increasing  
load.

From the theoretical considerations we  
derived the following relation between flow and  
load. The flow was decreased with increasing  
load. The flow was decreased with increasing  
load. The flow was decreased with increasing  
load. The flow was decreased with increasing  
load.

Mills, M. M., Oelrich, O., Rohrer, A., and KJ P.  
The purpose of the present study was to examine myoelectric  
function in the left ventricle of anesthetized open-chest dogs  
where localized part of the muscle mass was selectively  
stimulated with the  $\beta$ -adrenergic agonist isoproterenol. A  
stimulus was established between the left aortic artery and  
the anterior descending coronary artery for infusion of isopro-  
terenol. One pair of plane-electric elements was inserted  
into the area to be stimulated (the isoproterenol and the  
control area). The distance between the two plane-electric  
elements was continuously recorded by an ultrasonic  
technique to estimate muscle fibre function. Small doses  
of isoproterenol (0.1-0.5  $\mu$ g/min) which induced no general  
hemodynamic effects, elicited the contraction pattern of  
muscle fibres both in the stimulated and in the control area.  
In the control area, muscle fibres were stretched in early  
systole due to the greater rate and magnitude of tension  
development in the stimulated than in the non-stimulated  
muscle fibres. When the control area was later stimulated  
isoproterenol-stimulated, muscle fibres were stretched  
because they already were in the relaxation phase with low  
tension. On the other hand the control area  
contracted to lower MCL. Hence administration  
of isoproterenol to part of the left  
ventricle results in synergistic contractions  
and reduces the overall  
performance so that  
stroke volume may not  
rise despite extensive  
isotropic stimulation.

The influence of the interventricular septum on left  
ventricular function was studied in 7 anesthetized open-chest  
dogs during volume loading of the right ventricle. Myoelectric  
fibre function in the septum and in the free walls of the right  
and left ventricle was estimated by implanted ultrasonic  
elements. The distance between the elements is called  
myoelectric chord length (MCL). Volume loading of the right  
ventricle was achieved by opening a shunt between the  
pulmonary artery and the venous cava. By opening the shunt  
right ventricular stroke volume rose by more than 90%.  
End-diastolic MCL and systolic myoelectric shortening (MCL  
in the free wall of the right ventricle and in the septum rose  
whereas end-diastolic MCL and MCL in the left ventricular  
free wall decreased. Left ventricular stroke volume fell  
by 7-15%.

To determine the significance of stretching of the muscle  
fibres in the septum during right ventricular loading for left  
ventricular performance, venous flow was constricted until  
end-diastolic MCL of the left ventricle free wall was  
reduced as much as during volume loading. By venous  
constriction end-diastolic MCL of septum and free wall fell  
in proportion, and left ventricular stroke volume was reduced  
by 13-15% which is significantly more than 7-15% (P < 0.01).  
Thus volume loading of the right ventricle activates the  
Frank-Starling mechanism of the septum. The early  
performance of the left ventricle is also improved.



50

Sel th O R F M Vth Mo H d M g O D  
I stit to of Medl Biology M l alty O  
Trasse Norway) MODE OF ACTION OF ALYURONI  
BASE IN ACUTE MYOCARDIAL ISCHEMIA IN DOGS  
I d to t dy th echo is by whi b  
hyal idas red a my m di l i hant inaj  
y thre p t d c o y t r y l i  
ve p f read i 12 p h t sth tiz d  
daga T i ic th high pl s con t ti  
f fre fatty id (FFA) s lly B rv di  
t myocardi l i f ti i ma dan  
11 (0 125 ug/l kg) f f d i d i g  
th 2 d a 3rd l i My laro id 225 N  
unitt/kg wa gi f 20 n b fo the 3 d  
eccl i i hemic i ju s d o th  
em f ST egue t i tte (ZST) i pi d  
i ECR re d g from 10 16 it ft s d  
10 i oc l s R glo i myocardi l bl d  
ph r El d s a p d l belled ic  
f r y si (ca) d from lo l si (i)  
dral i g i hant ti d s d d i  
diff f q l i ct t FFA d O<sub>2</sub> were  
d t rat ed My l ro das treatment red d EST  
p 44 7 s d to 34 p 8 8 V (m) SEW  
p 0 005 t s i ccl sies a d fre 48 4 6 5  
to 39 6 7 i V (p 0 005) t 10 i u l to  
Th s 205 i s (p 0 01) i i hant  
epi rdial blood flow ft h y l ro id tre v  
best bat o ig ifi t h g a i flow i s  
ischmi d c rdus My l rend h d ig  
if i t i flu y rd l flu r b  
t t s d O<sub>2</sub> o t h hant  
t i s cl f ay l re id d ed i h  
mi i f ry d i g dren l f f i Th  
d tion may b p l d by i c ed 11  
t l bl od flow d t by l tr ad h g  
f b t t cro th i hant ay dion

51

Madhary A-1, Mikkelsen K P, and Molinoff P  
B 2) (Dept f Pharmacology AB 12 i Molndal  
Sweden 1) University of Colorado Medical Center  
Denver Col rado 2) DIFFERENTIAL DISTRIBUTION  
OF  $\beta_1$  AND  $\beta_2$ -ADRENERGIC RECEPTORS IN CAT AND GUINEA  
PIG HEART

Accumulating evidence indicates contradictory to the concept f Lands (1967) coexistence  
of  $\beta_1$  and  $\beta_2$ -adrenoceptors in the same organ  
(Carlsson t 1 1972) A difference in the  $\beta_1$ /  
 $\beta_2$ -ratio between the SA-node and the ventricle  
in the cat heart has also been discussed (Carl  
sson et al 1 1977) In the present study in diff  
erent  $\beta_1$  or  $\beta_2$ -selective ligands were used to  
inhibit the specific nonselective binding f  
125I iododihydroxybenzylindol 1 in crude mem  
brane preparation f right atrium (RA) and left  
ventricle (LV) f the cat and guinea pig heart  
Analysis f the data according to Hofstad revealed  
biphasic displacement kinetics indicative f  
binding to two receptor sites presumably  $\beta_1$  and  
 $\beta_2$  The regional mean relative densities f  $\beta_1$   
and  $\beta_2$ -adrenoceptors calculated from values ob  
tained with each competing ligand were as fol  
lows

	RA	LV	GUINEA PIG
$\beta_1$	78 1	98 0	76 7
$\beta_2$	21 9	2 0	23 3

The difference between RA and LV densities f  $\beta_2$   
adrenoceptors proved to be significant when cal  
culated (autosomes receptors/mg prot) The  
concentration of  $\beta_1$ -adrenoceptors in the cat LV  
proved to be nearly twice that in the guinea  
pig LV while there was very little difference  
in trial  $\beta_2$ -adrenoceptor concentration between  
the two species

Our data thus support the findings from the  
pharmacological in vivo experiments in cat

52

Romundt T, Ome J -B and Øye, I (Instit to  
f Pharmacology Uni f Os, Norway) ALPHA  
ADRENERGIC BLOCKADE IN RAT HEART BY PRazosin  
EVIDENCE FOR TWO ALPHA ADRENERGIC RECEPTORS

Phenylephrine (PE) in presence f prop amol  
ol (PNO) evokes an  $\alpha$ -adrenergic inotropic re  
sponse in mammalian heart The time-course f  
this response i characterised by transient  
decrease i maximal developed tension ( $T_{max}$ )  
f followed by slowly developing increase which  
reaches maximum after 4-5 min In t papil  
lary muscles PE (5x10<sup>-6</sup> mol/l) in pr sence of  
PNO caused transient decrease i  $T_{max}$  to  
88 % f control f followed by an increase t  
134 % of control

Prazosin (PZ) which i believed to be  
selective blocker f postsynaptic  $\alpha$ -receptors  
displaced the dose-response curve for  $T_{max}$  and  
d $T/dt_{max}$  to the right (ED<sub>50</sub> values 0 53 and  
0 57 respectively p<0 01) without change in  
maximal effect PZ thus behaved as competi  
tive antagonist with high affinity (inhibition  
constant ~ 4 10<sup>-6</sup> mol/l pK<sub>i</sub> ~ 6 4)

The effects of phentolamine (PT 5x10<sup>-6</sup> mol/l)  
and PZ (5x10<sup>-6</sup> mol/l) on the time course f the  
response differed qualitatively PZ blocked  
the positive component only (111 % of control  
p<0 01 compared to PE) f the inotropic re  
sponse whereas PT inhibited both the nega i ve  
(91 18 f control p 0 02 compared to PE and  
PE PZ) and the positive component (110 48 f  
control p<0 01 compared to PE)

These data may indicate that there re both  
inhibitory and excitatory  $\alpha$ -adrenergic recep  
tors in t heart located postsynaptically  
The excitatory receptors may be 1 due to the  
preference f PZ while the inhibitory ones  
may be 2 due to the non-selectivity action  
f PT

53

Kettunen R, Timisjärvi J, Kuikka J and  
Elonen, L (Department f Physiology Uni ve  
rsity f Oulu Finland) EFFECT OF EXERCISE AND  
SMOKING ON BLOOD CIRCULATION ASSESSED BY RADIO  
CARDIOGRAPHY

Radiocardiography provides self non in  
vive tool for determining functional blood vol  
ume flows f systemic and pulmonary circuit  
cardiac chamber volumes and for the mathematical  
estimation f pulmonary arterial mean and capil  
lary pressures permitting also exposure to var  
ious stimuli such as exercise and smoking We  
carried out an indirect or tachycardic radio  
cardiography on 40 volunteers (age 20 to 52 yr) i  
rest and during upright bicycle ergometer exer  
cise with and without smoking At rest the re  
sults obtained by repeated recordings were equal  
Smoking raised heart rate and aortic output  
but did not cause further significant changes  
Two different exercise series were carried out  
1) three repetitive recordings with the first at  
rest and the following 2 two level exer  
cise 2) two repetitive recordings with an in  
terval f 30 min the second work load preceded  
by smoking In series 1 cardiac output in  
creased up to 220 % of stroke volume 30 % ject  
ion fraction 15 % and pulmonary blood volume  
38 % The change could be depleted by poly  
methyl fucation with heart at as the abo  
Pulmonary capillary pressure decreased when car  
diac output increased In series 2 heart rate  
and pulmonary capillary pressure raised ig  
nificantly higher values fte compared to be  
fore smoking while decrease was observed in  
stroke volume pulmonary blood and dispersion  
volumes The conclusion i that smoking impairs  
physiologic performance increase pulmonary capil  
lary pressure decrease pulmonary blood volume  
and probably the number f open capillaries

Toivo M. Sakki, E. A. Vähitalo, E. and  
Hirvonen, A. (Mäkitie Central Hospital,  
75500 Lahti 65, Finland): LARGE FIELD GAMMA  
IMAGING IN STUDIES OF CERVICAL AND CEREBRAL  
CIRCULATION:

With a standard field gamma camera it is  
not easy to study the anatomic and functional  
status of the cervical and cerebral circulation  
simultaneously.

In order to examine the arterial circulation  
from aorta via cervical arteries to hemispheres  
in the same study a large field ( $\approx 40$  cm)  
gamma camera was used. A bolus injection of  
15 ml  $^{99m}\text{Tc}$  was used. By aid of a computer  
it was possible to get a visual impression of  
the status of various vessels in different  
phases of circulation. The regional  
circulatory curves were written bilaterally  
from the areas of common carotid arteries and  
cerebral hemispheres. The circulatory time  
from the aorta to the convexity was calculated  
too. This offered valuable further information  
especially in the diagnosis of cerebral AV-  
malformations and infarctions. The correlation  
of large field gamma images with x ray  
angiography was good.

**Conclusion:** The dynamic imaging of blood  
flow from aorta to cerebral hemispheres is  
possible with a large field gamma camera. By  
aid of the computer technique it is possible  
to get a visual impression of the status of  
various vessels and to calculate dynamic  
circulatory curve from the regions of interest.  
The large field gamma imaging seems to be a  
suitable noninvasive method for a routine  
in topographic laboratory.

Ilkka T O A (Department of Pediatrics  
University of Oulu, Finland): VECTORCARDIO-  
GRAPHIC PATTERNS IN IDIOPATHIC NORMAL VALUES AND  
INDIVIDUAL VARIATION

Frank y and leads were recorded from 62  
healthy full-term infants during the first 48  
hours and then the age of one week, 2, 4, 6,  
9 and 12 months with the equipment developed by  
Arvedson. The planar and partial QRS loops  
were analyzed by computer. The mean axis of  
the 5th and instantaneous vector and their 5th,  
10th, 50th and 95th percentiles were computed.  
The spatial vectors to the right and  
to the left, spatial mean QRS and ST T vectors  
and spatial angular deviation of T axis from  
QRS axis were computed.

The mean horizontal loop rotates clockwise  
at the age of one month and the figure-of-  
eight loops rotate counterclockwise.  
The initial vector is directed anteriorly to  
the right but its angle of one month  
to the right. Their magnitude increases during  
the first 6 months. The direction of ST and T  
vectors change very considerably during in-  
fancy from a right to a left. Their mag-  
nitude increases with age. There is little  
change in T spatial vectors.

The individual variation of the initial  
vector at 35 and 40 ms vector is very large  
and quite high in most than vector. The  
values of axis shift are more than the axis of  
deviation and magnitude.

It seems that cardiac electrophysiological  
change quite individually in time and in space  
during the first 12 months in infancy. It is  
great variability of normal values.

Timo Järvi J and Hirvonen L (Department of  
Physiology University of Oulu, Finland): THE  
BLOOD CIRCULATION OF THE REINDEER.

Right heart catheterizations with the recordings  
of a glass-catheterized aortic catheter and  
blood pressure were performed on 25 reindeer  
aged from 5 to 18 months. The calculation of  
left ventricular volumes utilized cut-down  
evolution ellipse as geometric reference  
and two separate regression lines for volume  
correction. The left ventricle contracted like  
blacksmith bellows. Stroke volume was 2.6  
ml/kg cardiac output 120 ml/kg/min. Ejection  
fraction 76%, heart rate 80 b/min, aortic  
pressure 133/130/115 mm Hg and pulmonary ar-  
terial pressure 25/16/11 mm Hg in the prone po-  
sition at rest. With the animal on its side pul-  
monary arterial pressure increased by about 10  
mm Hg, cardiac output and stroke volume by 25%.  
Artificial hypoxia increased heart rate, aortic  
and pulmonary arterial blood pressure and ejection  
fraction. If the circulatory variables were taken as discrete  
groups, correlation between them could  
be calculated. A second power polynomial  
correlation between a) heart rate and aortic  
blood pressure, b) heart rate and left  
ventricular volumes, c) aortic blood pressure  
and left ventricular volumes, d) aortic blood  
pressure and pulmonary artery pressure. In these experiments the reindeer  
cardiorespiratory system reacted readily and  
adapts to various stimuli. Its blood oxygen  
carrying capacity was high (180 g/l blood  
volume 106 ml/kg) and there existed high  
degrees of cardiac reserves. The pulmonary  
circulation appeared to be suitable model for  
study of pulmonary hypertension.

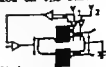
Sebastian M (Department of Biophysics Univer-  
sity of Copenhagen, Denmark): MEMBRANE POTEN-  
TIAL CONTROL IN MAMMALIAN HEART MUSCLE

Voltage clamp studies on cardiac muscle  
utilizing the sucrose gap technique are compli-  
cated by diffusion in the extracellular space of  
sucrose into the compartments perturbed with  
physiological salt solution and vice versa.

Another complication stems from the superfi-  
cially located cells in the potential control  
lead part of the preparation having smaller  
series resistance to ground than the deeply lo-  
cated cells. Thus radial potential gradients in  
the extracellular space will compromise the ho-  
mogeneity of the potential control.

Experiments on papillary muscle or trabec-  
ulae carneae from guinea pig, cat, pig and dog  
reveal that the sucrose gap can be replaced by  
a controlled localized compression of the ex-  
tracellular space effected by reducing the  
diameter of hole in 3 mm thick rubber par-  
tition through which the preparation has been  
pulled. This way the longitudinal resistance  
can be increased to 10-20 M $\Omega$  before the  
intracellular resistance is influenced.

Radial potential gradients in the extracel-  
lular space of the potential controlled and  
the preparation are diminished by reducing the  
amount of the surrounding salt solution to  
thin (10  $\mu$ m) flowing layer creating an arti-  
ficial extracellular space on the surface of  
the preparation. With  
one of the current elec-  
trodes positioned at the  
end of the preparation  
superficial and deep  
cells will have equal  
membrane current density ( $I_m$ )





Ask J, A. Stene-Larsen G, and Well K.B (Institute of Physiology RRI Univ of Bergen Norway) THE CARDIAC  $\beta_2$  ADRENOCEPTOR IN THE RAINBOW TROUT

We have studied the adrenoceptor-mediated responses in the heart of trout with  $\alpha_1$  and  $\alpha_2$  adrenalinic (A) in the sympathetic terminal of the rainbow trout. The chronotropic and inotropic responses recorded isometrically in the spontaneously beating auricle *in vitro* were maximal at 170 and 14 respectively. The force and frequency were potentiated by salbutamol (sal) at 2.14 C while neither of the selective  $\alpha_1$ -adrenoceptor agonists had any effect at this temperature interval. At 8 C the  $\alpha_2$  agonist by affinity was Isoprenaline > A2 sal > noradrenaline (NA) > the chronotropic and inotropic response. The same order was obtained for the maximal effects of the agonists on the isometric responses while for the frequency the order was A NA > Isoprenaline sal. The  $\beta_2$  antagonist H15 Le 35/25 was the only one of the selective  $\alpha_1$  and  $\alpha_2$  receptor blockers with effects on the trout heart at 2.14 C.

Hence the trout heart is controlled via  $\beta_2$ -type of innervated cardiac adrenoceptor

Wohlfart B and Jönas K.A.P. (Department of Pharmacology University of Lund Sweden) AN ANALYSIS OF THE EFFECTS OF DIGITALIS ON THE EXCITATION-CONTRACTION MECHANISM OF MAMMALIAN MYOCARDIUM

Previous experiments have shown (Edman & Johansson, J Physiol 1976 254 545-561) that maximal myocardium produces optimum contractility (OCC) at 0.8 Hz.

After preceding stimulus and that the contractile strength thereafter slowly decays. Peak force of OCC can be quantitatively predicted (Wohlfart, Acta physiologica 1979 in press) by the following equation:

$$OCC = AP \cdot f \cdot \gamma$$

In this equation AP and f denote action potential duration and peak force respectively,  $\gamma$  the maximal preceding contraction and  $\beta$  and  $\gamma$  are constants expressing the inotropic state. The constant probably reflects the intensity of the transmembrane flow of activator calcium during the action potential and the degree of reutilization of calcium released during the preceding contraction.

Isolated papillary muscles of the rabbit were paced to contract isometrically at basal frequency of 0.67 Hz (37°C). AP and f were varied by introducing an extra beat and by varying the interval between the extra beat and the contraction preceding OCC. Ouabain (0.3  $\mu$ M) did not affect the interval (0.8 s) required to elicit OCC. The glycoside potentiated OCC, caused marked increase in f and less pronounced increase of  $\beta$ . The positive inotropic effect of digitalis is therefore most likely attributable to an enhanced transport of calcium into the cell during the action potential and to more efficient reutilization of the calcium that is released from the cell during relaxation. In high concentrations digitalis may also enhance the influx of calcium through the cell membrane between action potentials. This is indicated by the finding that 8.0  $\mu$ M ouabain increases the resting-state contraction, i.e. the contraction elicited after long (20 min) pauses.

Hollner P, B. Wohlfart and B. Jönas (Department of Pharmacology University of Lund Sweden) EFFECTS OF 4-AMINOPIRIDINE ON ISOLATED PAPILLARY MUSCLES OF THE RABBIT

Microscopic concentrations of 4-aminopyridine (4-AP) have been shown to greatly potentiate the release of transmitter at the neuromuscular junction. There is no evidence for an increased release of transmitter in adrenergic nerves. These effects have been attributed to an increase in action potential duration caused by an inhibition of the repolarizing  $K^+$  current. Higher concentrations (1 mM) of 4-AP slowing the action potential and potentiated the isometric twitch in amphibian and mammalian skeletal muscle fibres probably by increasing the intracellular release of activator  $Ca^{2+}$ . In the present study the effects of 4-AP were investigated in rabbit papillary muscles at 37°C. Membrane potentials were recorded simultaneously with isometric force production. 4-AP in a relatively low concentration (0.01 mM) gave transient increase in force development which was counteracted by propranolol and therefore considered to be caused by noradrenaline release. At higher concentrations 4-AP (1 mM) the presence of propranolol had little or no effect on the inotropic effect which reached an optimum (20-40% increase in peak force) at 0.1-0.2 mM. The inotropic effect was associated with an increase in the time to peak force and time to peak force and time to half relaxation. The action potential progressively increased in duration over the range of concentrations (0.01-2 mM). The results are explainable by assuming that 4-AP inhibits the repolarizing  $K^+$  current (cf. above). The positive inotropic action may be due to prolongation and possibly also to an intensification of the  $Ca^{2+}$  influx during the action potential. A similar mechanism has been proposed for the nerve terminal.

L. Björkman, J.-A. Carlsson & Carlsson K. (Pharmacology Dept. AB Hassle Mölndal Sweden) CARDIAC INOTROPIC EFFECTS OF PRENATALOR AND OUABAIN IN CONDUCTION DOGS

Prenatalor an  $\alpha_1$  active  $\beta_1$  selective adrenoceptor agonist has been shown to elicit more marked stimulation of cardiac isometry than chronotropic when compared to isoprenaline (Carlsson et al. Scand J Clin Lab Invest 1977). The finding made it of interest to study the haemodynamic effects of prenatalor (P) and ouabain (Ou) given alone or in combination. Beagle dogs chronically instrumented with flow catheters around the ascending aorta and an intratrial catheter were used. ECG stroke volume (SV), aortic flow acceleration (max dP/dt) and mean aortic blood pressure (MABP) were recorded. P (45 nmol/kg) and Ou (50 nmol/kg) were given i.v. and the effects were recorded 2-5 min later. The effects expressed as percent differences compared to saline control values were as follows:

Substance	HR	MABP	SV	Na	dP/dt	PO	intact
P	+1	0	23	31	31	-8	
Ou	1	9	13	23	9		
P + Ou	1	3	24	59	-4		

No arrhythmias were observed. The data show that P significantly augments cardiac contractility with unchanged HR in the conscious dog. The inotropic effects of Ou and P seem to be additive which may imply that P in combination with digitalis glycosides may be of beneficial effects in the treatment of heart failure.





Svensson, T.H. and Thörén, P. (Departments of Pharmacology and Physiology University of Göteborg, Sweden) LOCUS COERULEUS NEURONS: VAGALLY MEDIATED RESPONSE TO ALTERED BLOOD VOLUME

Several previous studies suggest that the pontine noradrenergic nucleus locus coeruleus (LC) may participate in maintenance of sympathovagal balance and arousal as well as in the regulation or modulation of autonomic and cardiovascular function, including various reflexes. Thus, electrical stimulation of the LC producespressor responses and recently afferent stimulation of the vagus nerve was found to cause inhibition of LC neuronal activity. To further characterize the role of LC neurons in autonomic function we have used single cell recording techniques to study in the rat the LC response to physiologically relevant stimulus for cardiovascular reflexes, namely volume load. The intravenous injection of 1-4 ml blood or dextran (Macrodex 10%) caused volume-dependent reduction in firing rate of LC neurons. Withdrawal of corresponding amount of blood caused prompt return to baseline activity. Also bilateral vagotomy in the neck produced this effect. Thus, LC neurons respond reflexively to moderate alterations of blood volume. This effect is in all probability mediated via vagal efferents, presumably activated from arterial receptors.

(Supported by the Swedish Medical Research Council)  
proj. Nos. 4747, 4764 and 0016)

Lassnig, R., Lassnig, E., Pekkari, J. and Leppä, M. (Department of Pharmacology and Dermatology University of Turku, Finland) BENIN IN HYDROCORTISONE HYPERTENSION OF RATS AND IN PREDNISONE THERAPY OF PATIENTS

Hypertension was induced in adult rats by hydrocortisone (20 mg/kg in drinking water or tail dipped daily in glycerol containing 2.5 mg/ml hydrocortisone) with 2% saline as drinking water. 300 µl blood was sampled from tail vein 2 weeks 0, 2, 4 and monthly for radioimmunoassay of plasma renin activity (PRA). Blood pressure (BP) of 150 mmHg was reached in 4-8 weeks. In 4 treatment series PRA rose rapidly 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100. In 23 weeks PRA rose at least 10 months (n=45, p<0.001). Propranolol (80 mg/kg) and metoprolol (100 mg/kg) in drinking water prevented hypertension below 125 mmHg, and PRA did not change from the pretreatment level (n=29). Drinking 2% saline increased BP gradually up to 125 mmHg in 16-20 weeks whereas PRA decreased to half transiently after the first 8-12 weeks (n=37, p<0.01). DOCA (1.25 mg/kg) with line drinking induced hypertension up to 175 mmHg with a half decrease in PRA to one tenth from the basal level (n=10, p<0.001).

Prednisone therapy (30-40 mg daily) was studied in 13 patients hospitalized for the treatment of dermatological diseases. No other systemic drug therapy was given. BP was found normal during therapy. PRA increased by 4% in 3 days and after 2 weeks therapy PRA was 124% above the control level (n=5, p<0.05).

PRA in hydrocortisone-saline hypertension reflects more glucocorticoid than mineralocorticoid mechanism. Thus in hydrocortisone-induced hypertension and in glucocorticoid therapy angiotensin can be contributing factor to hypertension.

Olsson, R., and Nilsson, L. (Department of Clinical Physiology and Radiology, University of Turku, Finland) RADIOGRAPHIC STUDIES IN ALLERGIC ALVEOLITIS

Allergic alveolitis is rather common among the rural population of Eastern Finland. This is clearly correlated with the handling of moldy hay during winter season.

46 subjects with the mean age of 47 yrs (21-66 yrs) in acute or recovery phase of this disease were studied with common lung function measurements and with <sup>125</sup>I-indium radioangiography (RCA) test.

RCA parameters: stroke volume index (SVI), left ventricular ejection fraction (LVEF), pulmonary blood volume index (PBVI) and pulmonary capillary pressure (PCP) were compared to the corresponding figures of 46 control subjects. The results are given in the following table.

	SVI	LVEF	PBVI	PCP
mean	52	0.62	265	8.9
S.D.	13	0.11	60	1.6
number of pathological findings	3/46	6/46	19/46	11/46

A confidence limit for pathological findings is 95% (control mean  $\pm 2$  S.D.). The stroke volume and pulmonary blood volume indices correlated significantly (p<0.01) with the arterial blood oxygen partial pressure (ABPO<sub>2</sub>) (r=0.41 and r=0.47 respectively) but not with the diffusion capacity of lung (DLCO). There was nearly significant correlation (p<0.05) between the heart rate and ABPO<sub>2</sub> (r=0.31).

The most promising RCA-index seems to be PBVI but further extensive experiments are required to prove the usefulness of RCA-studies.

Kka, J.T., Linninen, E., Pihlén, M.A., K.O. and Valtio, R. (Department of Clinical Physiology and Lung Diseases, University Center Hospital, Kuopio, Finland) RADIOGRAPHIC STUDIES IN ALLERGIC ALVEOLITIS

Allergic alveolitis is rather common among the rural population of Eastern Finland. This is clearly correlated with the handling of moldy hay during winter season.

46 subjects with the mean age of 47 yrs (21-66 yrs) in acute or recovery phase of this disease were studied with common lung function measurements and with <sup>125</sup>I-indium radioangiography (RCA) test.

RCA parameters: stroke volume index (SVI), left ventricular ejection fraction (LVEF), pulmonary blood volume index (PBVI) and pulmonary capillary pressure (PCP) were compared to the corresponding figures of 46 control subjects. The results are given in the following table.

	SVI	LVEF	PBVI	PCP
mean	52	0.62	265	8.9
S.D.	13	0.11	60	1.6
number of pathological findings	3/46	6/46	19/46	11/46

A confidence limit for pathological findings is 95% (control mean  $\pm 2$  S.D.).

The stroke volume and pulmonary blood volume indices correlated significantly (p<0.01) with the arterial blood oxygen partial pressure (ABPO<sub>2</sub>) (r=0.41 and r=0.47 respectively) but not with the diffusion capacity of lung (DLCO). There was nearly significant correlation (p<0.05) between the heart rate and ABPO<sub>2</sub> (r=0.31).

The most promising RCA-index seems to be PBVI but further extensive experiments are required to prove the usefulness of RCA-studies.



TS d 11 A R d S ds E (Dep rtements of  
Physi logy d Pathol gy i att ite f Medical  
Bi logy U iversity f Tromsø Norway) THE  
INTIMAL SURFACE OF THE RABBIT AORTA  
By ne f isec s two p e t a tting  
nat 11 (11g 1 504) h y de ta of  
abhit o t s t pr s res varying f om atm s  
ph i E ab yet lic We mo tored the  
dit u l of the t by suring it di  
t u l a eptic l le system s dw  
blecked all b h t r i s by 3 phad sph s  
W f i d f l d e p s at on th i t i l  
s f t g t or less r t e t at 11  
deg e f d ite to

Sovijärvi A R A No set S Ryvärinen J  
Fran il A G raeff M and Lahtimäki M  
(Departme t f Physiology U niversity f Waisla  
hi and Departme t f Electrical Engineering  
Tampere University of Technology Finland)  
EFFECT OF AIR IONIZATION ON HEART RATE AND  
PERCEIVED EXERTION DURING EXERCISE

The influence f ai ion t ion on heart rate  
(HR) and ting f pe ceived exertion (RPE)  
during bicycl ex rcise was studied in lse  
healthy medical todent andonly selected from  
the lase f 90 students The exercise test  
were pe f rmed both unde negative and positive  
ion t ion The study wa med with double-  
blind ro over de ign The body surface  
xposed t ion current was made large by  
reducing the l thing f the subject A  
ignifi ant over-all tendency to lower HR and  
RPE values under negative ion t ion wa ob-  
served (P<0.01 ign test) The RPE values  
were ignifi antly lower (P<0.01 paired t  
test and th Wilcoxon test) under negative  
then unde positive ion t ion t the maximal  
wo k load level However HR values did not  
diff ignifi antly under negative and posi-  
ti ionisation t any lative load level  
The sult f this pilot tudy indicat  
that ion composition of air an modify the  
RPE and pos ibly lso HR during exercise; nega-  
tive ion t ion seems t be benefi il compared  
with po itive ion t ion The mechanisms in-  
volved re becaue but we ggest that nega-  
tive ion t ion of ir may incr ase oxidative  
metabol sm through gene t ion f superoxide  
radical (O<sub>2</sub>) that i reduced = H<sub>2</sub>O<sub>2</sub> by super-  
oxide dismutase

Huber W and Secher, H R (Ca th n tional Is-  
chool t on f I f il Paraly and Medi al  
departme t f Bi ophysi cs Ho pital Denma k)  
FORCE TIME INTEGRAL OF FAST AND SLOW TWITCH MUS-  
CLE FIBERS AT ONSET OF EXERCISE

3 healthy y ung mal bje t performed 10 re-  
peated max mal voluntary ontra tions with fleg-  
ga muscl once very fifth d Erik ocu-  
t t on were ephal ied I th meanagress  
f t d low component could be parated  
f ro time i t g rai (f TI) for th two compo-  
ne t we d t rai d during the fi t 600 ms f  
h t t on  
T me we mea for g for the f t nd the  
low compo t 202 69 d 590 52 (S E)  
me pe ti ly Th fast compo t had d  
rat on f 337 4 ms whil th low component  
could may t i for f 1 nd f TI  
as 9.3 0.29 and 42.7 0.51 M f the  
f t nd th low component t re pecti ly The  
eo respond g ma l us f ro were 51.7 ± 0.39  
nd 102 8.6 M f TI f the f t compo t had  
t l g t rpl t d ft 136 ± 3.7 ms Doried  
the 10 MVC re d rding the rel ti f TI f lse  
eomponen di an ed coordi g t the quenc  
98.9 2.95 1 0.997 ( d i gnat th mme-  
be f t raction) f TI f th f t compo t  
did t change (P 0.9)

E s g r ted that the f t and low com-  
pone t repre ent th me ogr ma f f t nd  
low l h mu l f b rma re pecti ly f  
h f t t it h us l f b ha f i d  
th low t it h usal fibe ha t ni oer-  
tra t ion patt i humans f tigue i repe ted  
me amal lunt y ontra t ion may in l only  
the onl oo ion lense t

CHANGES IN MYOGENESIS AND  
TRACE LULAS WUS  
TE CONTEXT AM  
MA OR INTX  
i 11 d we d h vy  
nd k f l ma p us  
i i g l nd h pl sm  
g i i d tr 11 ar  
d i d  
al by me pe f rmed 3 ho ta f j i  
i ioy l d y  
pe od R p (f  
me 1 ps h ) nd f blood  
pl b be d  
11 far mi ed  
Th di abu i pa (jag 9.9  
(i ed f p (used nc ed 0  
( ) meol /l f om 55 2 )  
me /l d ro 0 91 (98) 0 ( i l sm l  
Pg/l i Le i f t ow mg g  
f seed f 3.3 151 359 / d  
tr i f ( ) d  
0.19) 1/10 g 1 om 9.8 ( g  
is 5.5 ) meol/100 g d  
ce dec ed f em )  
11 (13) me /l fi  
f 3 i Tr y h i hang  
d 11 lyt eo  
1eq me h y aff me  
i k i  
S; g ar G ( 8.9) Un Copenh g

**Secher, H. H.** (Department of Anaesthesia, H. R. Hospital and August Krogh Institute, University of Copenhagen, Denmark) **FORCE PATTERNS DURING REPEATED MAXIMAL VOLUNTARY CONTRACTIONS**  
 A healthy male subject performed repeated maximal voluntary isometric contractions (MVC) with arm, hand, finger muscles on very ten seconds for 130 to 1600 times in order to record the contraction pattern in figure 1. The force recordings two maxima were reversed one after the other (17, 18) the after 12 (80, 24) (median value with 95% confidence limits). With repeated effort force and EMG amplitude decreased and the duration of the force performance decreased from 2.3 to less than 1.5 with only one maximum in force. Changing from blind led to the performance of the task resulted in marked increase in force amplitude and re-appearance of two maxima contraction pattern. The early and late maxima are interpreted to reflect the contraction times of the fast (FT) and slow (ST) muscle fibres respectively. Thus it is suggested that the FT fibres are decisive for the force of the contraction in MVC while the ST fibres maintain force. It is further suggested that ST fibre involvement is decreasing with repeated maximal effort but that the ST motor unit can be partly recruited with diverting activities (Batchesova phenomenon).

**Peterson, S. and Secher, H. H.** (Medical Department P, Bispebjerg Hospital and Danish National Association of Infantile Paralysis, Copenhagen, Denmark) **FORCE-TIME INTEGRAL OF FAST AND SLOW MUSCLE FIBRES IN FATIGUED MYASTHENIC PATIENTS**  
 Repeated maximal voluntary contractions were performed with finger muscles once every five seconds for eight minutes in four control subjects and in four myasthenic patients. Force-time integral (FTI) was determined during the first 1.25 s of the contraction.  
 FTI decreased by 30% in the control subjects and by 65% in the myasthenic patients. In the mechanograms, fast and slow components could be separated. Time to maximum force was 160 ms and 480 ms in the fast and slow components respectively. The duration of the fast component was 320 ms and it did not change with repeated effort. The slow component had duration of several seconds. In control subjects FTI of the fast and slow components was 6.6 and 75 Nms respectively. FTI of the fast component remained constant while FTI of the slow component decreased to 45 Nms in figure 1. In myasthenic patients FTI of the fast component was 5 Nms and did not change with repeated effort. FTI of the slow component decreased from 30 to 8 Nms. Prednisolone treatment had no effect on FTI of the fast component but increased FTI of the slow component by 250-300%.  
 It is suggested that the fast and slow components represent the mechanograms of the fast and slow twitch muscle fibres respectively. Thus fatigue in repeated maximal effort may involve low twitch muscle fibres only. In conclusion myasthenia gravis is a disease of abnormal low twitch muscle fibre fatigue.

**Jørgensen-Kjaer, T., Bakker-Kristensen, S., and Jørgensen, N. V. and Secher, H. H.** (Danish Clinical Research Center and Cardiology Lab, Med Dept B, Rigshospitalet, Copenhagen, Denmark) **CENTRAL CIRCULATION AND PLASMA CATECHOLAMINE RESPONSES TO ONE LEGGED EXERCISE IN MAN**  
**INFLUENCE OF UNILATERAL MUSCULAR HYPOTROPHY**  
 Exercise with hypotrophic muscles shows different cardiovascular and metabolic responses as compared to work with normal muscles point to peripheral factor modulating exert responses.

Circulatory and metabolic responses were investigated with the one-legged exercise model using 3 young healthy subjects who for 5 weeks had one leg immobilized in cast. Each leg was investigated separately after the immobilization period during graded submaximal and maximal bicycle work. Maximal oxygen uptake was 2.37 and 2.18 (l/min) respectively for the weak and the control leg. Heart rate and cardiac output was linearly related to VO<sub>2</sub> for both legs. However, the slope for the weak leg was positioned significantly (p < 0.05) higher than for the control leg. Noradrenaline increased from 0.25 to 2.18 ng/ml for the weak leg and to 1.42 for the control leg. The same curvilinear relation was observed for the same cardiovascular relation. The correlation coefficients were found between mixed venous oxygen saturation and noradrenaline, no difference was obtained between the two legs (r = 0.94 and 0.91). Hypotrophic muscle induces greater central circulation during dynamic work than normal muscle and its reaction seems not related by plasma catecholamines.

**Hart, H. H. E.** (Department of Physiology, University of Tampere, Finland) **PHYSICAL TRAINING UNDER THE INFLUENCE OF BETA BLOCKADE IN RATS**

I have been very interested in the effects of physical training on the sympathetic nervous system during the rat period. If this hypothesis is valid, then physical training would influence the beta blockade should lead to a definite change. This situation is very common in men who are dependent on long-term drug therapy with beta-blockers and who are recommended limited physical training. The rehabilitation program of this study was combined effect of physical training and beta-blockade have been investigated in rats.  
 Rats were trained by daily running and swimming for 4 to 8 weeks. Both training and beta-blockade led to cardiac enlargement and lower resting heart rate. Increased heart rate response to isoprenaline, increased sensitivity to the oxidized adrenaline in the tail and low heart rate during the training and low heart rate during the beta-blockade. However, only swimming training showed an increase in heart rate response to isoprenaline. All the above mentioned changes were smaller in the animal group trained and beta-blockaded than in the group trained and not beta-blockaded. The present results emphasize the importance of the sympathetic nervous system in the induced adaptive changes. If the sympathetic influence is prevented by beta-blockade, the changes commonly considered to be induced by physical training will develop in the animal.





Kauppinen, W. A. K. (Dept. of Physiology, University of Helsinki, Finland)  
REACTION OF RESPIRATORY AIRWAYS TO CO<sub>2</sub> EXPOSURE AT 10°C

1. cold, viscous mucus, and pro-  
duced a bronchospasm. The effect was  
abolished by atropine. The effect was  
not observed in the control group.  
The study was undertaken to examine the influence  
of the respiratory tract on the respiratory function.  
In preliminary experiments, 6 healthy adult  
female swine were examined (1) in a room temperature  
and (2) in a room at 10°C. The blood  
pressure was measured at actual temperature and  
kinematic viscosity of the mucus was recorded.  
With a pneumograph, the following  
respiratory function was determined: FVC  
FEV<sub>1</sub>, MMEF, PEF, P50, V<sub>max</sub> 25, inspiratory  
rate and expiratory volume and plethory  
frequency.

On the average, 11 respiratory parameters  
determined were: tidal volume, respiratory rate, plethory  
rate, FVC, FEV<sub>1</sub>, MMEF, PEF, P50, V<sub>max</sub> 25, inspiratory  
rate, expiratory volume, and plethory frequency.  
The mean values in the control group were:  
tidal volume 1.5 l, respiratory rate 12 l/min, FVC 2.5 l,  
FEV<sub>1</sub> 1.5 l, MMEF 1.5 l, PEF 1.5 l, P50 1.5 l, V<sub>max</sub> 25 1.5 l,  
inspiratory rate 1.5 l/min, expiratory volume 1.5 l,  
and plethory frequency 1.5 l/min. The values in the  
10°C group were: tidal volume 1.5 l, respiratory rate 12 l/min,  
FVC 2.5 l, FEV<sub>1</sub> 1.5 l, MMEF 1.5 l, PEF 1.5 l, P50 1.5 l,  
V<sub>max</sub> 25 1.5 l, inspiratory rate 1.5 l/min, expiratory volume 1.5 l,  
and plethory frequency 1.5 l/min. The values in the  
10°C group were: tidal volume 1.5 l, respiratory rate 12 l/min,  
FVC 2.5 l, FEV<sub>1</sub> 1.5 l, MMEF 1.5 l, PEF 1.5 l, P50 1.5 l,  
V<sub>max</sub> 25 1.5 l, inspiratory rate 1.5 l/min, expiratory volume 1.5 l,  
and plethory frequency 1.5 l/min.

The results of the study show that the respiratory  
function is not affected by the cold exposure. The  
results of the study show that the respiratory  
function is not affected by the cold exposure.

CL EN(1949) J Phy 1(London) 109 421 9

Lagerstedt, K. J. H., Repo, T. and Saarimäki, K.  
(Biophysical Laboratory, Department of  
Zoology and Archipelago Research Institute,  
University of Turku, Finland) MA AND K  
ACTIVATED ATP-ASES IN THE GILLS OF MUSSELS  
FROM FRESH AND BRACKISH WATER

The rate of active transport of ions are  
not known in bivalve molluscs. However, micro-  
somes from the gills of the freshwater mussel  
*Anodonta cygnea callensis* show Mg<sup>2+</sup>-dependent  
Na<sup>+</sup> K<sup>+</sup>-stimulated as well as Na<sup>+</sup> and K<sup>+</sup>  
stimulated ATPase activity (Lagerstedt, K. and  
Saarimäki, K. Comp Biochem Physiol 1979 229,  
291). It is thus possible that the gill epithelium  
is involved in the ion uptake of these animals.  
In this study the ATPase activities were studied  
in membrane preparations from gill of *Mytilus edulis*  
adapted to brackish water with salinities of 6 or 15 o/oo.

Homogenates prepared from gill of *Mytilus*  
adapted to 6 o/oo showed monovalent cation  
stimulated ATPase activity which was about 1/3  
of that found in *Anodonta*. Na<sup>+</sup> and K<sup>+</sup> activa-  
ted but ouabain-insensitive. Depending on the  
assay conditions, the enzyme activity found in  
gill homogenates from *Mytilus* adapted to 6  
o/oo was 3-10 times higher than low Na<sup>+</sup> or K<sup>+</sup>  
stimulated ATPase activity was also observed.

Similar results were obtained with microsomes.  
As the activities of membrane ATPases in the  
gills of *Mytilus* are affected by environmental  
salinity, they probably participate in the ion  
regulation in this species. It can be suggested  
that in higher salinities more K<sup>+</sup> is exchanged  
for Na<sup>+</sup> by the high activity of the Na<sup>+</sup> and K<sup>+</sup>  
activated enzyme. In the freshwater mussel  
*Anodonta*, the Na<sup>+</sup> K<sup>+</sup>-activated enzyme is  
probably involved in the transport of other  
cation from the dilute environment.

Lehtonen, A. (Department of Diagnostic Radiology,  
University of Oulu, Finland) CITERADIO-  
GRAPHY IN THE EXAMINATION OF PHARYNGEAL LAR-  
YNGEAL AND TRACHEAL FUNCTION (Video)

With cine radiography it is possible to examine  
even rapid function.

Swallowing begins as an act of function of  
the mouth and tongue and continues as reflex  
function of the pharynx. The swallowed  
food as medium passes through the pharynx in  
about half second and the upper oesophageal  
sphincter opens about one second. By func-  
tion can be seen the function of the mouth  
in the contraction of the pharynx. The upper  
oesophageal sphincter he losing of the  
larynx during swallowing.

Larynx movement can be seen quite well  
even without contrast medium but the best method  
is to make cine radiography on using contrast  
medium (line laryngography). Paralysis of the  
recurrent nerve is the most common function  
disorder of the larynx.

Movement of the trachea can be seen  
in laryngography. In about one second  
during the respiratory function and cough the  
tracheal lumen becomes narrower than in inspi-  
ration but normally no more than half of  
inspiratory lumen. In tracheomalacia trachea  
becomes narrower than half of inspiratory  
lumen in severe cases.

In this demonstration normal laryngography  
of the pharynx, larynx and trachea and the most  
important functional disorders are shown.

Berglin, T. and Öbrink, K. J. (Department of Physiol-  
ogy and Medical Biophysics, University of Uppsala,  
Sweden) HISTAMINE AS A NORMAL MEDIATOR IN GASTRIC  
SECRETION

With the use of isolated glands from the gastric mucosa  
of rabbit, a new tool to analyze the stimulation  
procedure of the gastric cells is available. The glands  
respond to histamine and cholinergic drugs but not to  
gastrin. This may be due to destruction of gastrin  
receptors but could also reflect the normal absence of  
such receptors. In that case the action of gastrin in  
vivo would depend on liberation of histamine which in  
turn would stimulate the gastric cells. In the glandular  
preparation the liberated histamine would be enormously  
diluted and would not reach concentrations high enough  
to stimulate the gastric cells.

Histamine liberation has been analyzed after stimu-  
lation with pentagastrin and dose-effect curve was ob-  
tained with an ED50 of 1 nM pentagastrin. The lowest  
effective concentrations of pentagastrin were 10 to  
100 pM. The highest concentration of histamine liber-  
ated was below 0.1 nM which is the concentration  
barely detectable as giving secretory response in the  
gland. Thus it has been possible to separate the two

proposed sequences, namely the effect of histamine  
to liberate histamine and the effect of histamine  
to stimulate gastric secretion. The conclusion is that  
at least in the rabbit histamine is the final mediator  
for the action of gastrin on acid secretion.

1 Berglin, T. and Öbrink, K. J. Acta Physiol Scand  
22: 103-109, 1976

108

Andell, G., Sjöstrand, E., Olbe, L., and Nyberg, B. (Dept. of Pharmacology, Ås, Hålsjö, Malmö). **PHARMACOLOGICAL INHIBITION OF GASTRIC ACID SECRETION BY A NEW MECHANISM.**

A group of substituted benzimidazole has been shown to inhibit gastric secretion. They are more potent inhibitors of acid secretion and have much longer duration of action than the histamine  $H_2$ -receptor antagonists.

The inhibitory effects on gastric acid secretion by substituted benzimidazoles and the  $H_2$ -receptor antagonist metiamide were compared in vivo and in vitro in various experimental models.

In vivo studies in the dog and in vitro studies on the isolated gastric mucosa of the guinea pig have confirmed that metiamide inhibits the histamine stimulated acid secretion by competitive interaction. The substituted benzimidazole on the other hand showed non-competitive inhibitory action with dose-dependent reduction of the maximal acid response to histamine.

Histamine stimulated secretion in the isolated gastric mucosal preparation was inhibited by both metiamide and benzimidazole. Secretion stimulated by dibutylpyridyl AMP was equally inhibited by the benzimidazoles while metiamide had no effect.

In conclusion the mechanism of action of the substituted benzimidazole, in inhibiting acid secretion, differs from that of the  $H_2$ -receptor antagonists.

The substituted benzimidazole seems to interfere with secretory mechanisms peripheral to the  $H_2$ -receptor and peripheral to the cytosolic step with the parietal cell.

107

Glise, H., Abrahamsson, M. and Lindahl, B. (Department of Physiology, University of Göteborg, Sweden). **SEGMENTAL DIFFERENCES IN REFLEX ADRENERGIC INHIBITION OF GASTRIC MOTILITY.**

Local captive tiamil applied anywhere in the gut, i.e. its widespread adrenergic gastrointestinal inhibitory spinal reflex, but little is known to what extent such sympathetic-reflexes are segmentally different. This problem was studied in anesthetized and vagotomized cats using an indicator gastric motility continuously recorded by volume method while identical nociceptive stimuli were applied to the wall of consecutive gastrointestinal segments.

Nociceptive stimulation of the small intestine or proximal colon (splenic supply) caused prompt and profound antrectal relaxation, while similar stimuli to the distal colon (lumbar colon c. nerve supply) led to considerably weaker gastric inhibitions. The same difference was seen when tested identical electric afferent stimuli were applied to the mesenteric plexus destined to the small intestine or proximal colon and to the distal colon respectively. The reflex latencies (arterial pressure) were however equal whether proximal or distal GI segments or afferent nerve segments were stimulated. All reflex responses were blocked by ganglionectomy or by spinal anesthesia while they were unaffected by sympathectomy. Thus local captive stimulation of proximal GI parts with splenic nerve supply produces more pronounced reflex inhibitions of gastric motility than similar stimuli of distal colon mainly innervated by the lumbar colon nerves. This difference suggests considerable segmental differentiation of the spinal reflex centres for these inhibitory reflexes. It cannot be ascribed to variation in GI tone sensitivity, first because the results were the same when instead the afferent fibres were directly stimulated and, second, because reflex blood pressure responses were identical whether proximal or distal segments were stimulated.

108

Yam, W.-C., Department of Physiology and Medical Microbiology, University of Uppsala, Sweden. **STIMULATION OF  $HCO_3^-$  TRANSPORT IN ISOLATED ROSTA PROCTODUODENUM BY SOME ULCEROPROTECTIVE SUBSTANCES.**

Recent studies have suggested that the prostaglandins  $P_1$  and  $P_2$  (dibutyl-2) protect the gastric mucosa from damage by (acetylsalicylic acid) by stimulation of the active  $HCO_3^-$  transport in fundus and antrum. Some other ulceroprotective prostaglandins ( $G_1$  and  $G_2$ ) seem to act by inhibition of fundic  $H^+$  secretion. Antitrophic effects of prostaglandins have however been demonstrated also in the duodenum although no increase in mucosal secretion of neutralizing  $HCO_3^-$  seems to occur. Therefore it was of interest to study the effects of some prostaglandins on duodenal mucosal  $HCO_3^-$  transport.

The latter is very probably due to active transport originating from surface epithelial cells. Isolated bullfrog duodenal preparations devoid of Brunner's gland were used in the present study. The bulb and adjacent part of proctoduo duodenum (exposed length 12 mm) was dissected free from mesoenteric layers and mounted as tube between two glass tubes connected to reservoir.  $HCO_3^-$  transport into the luminal solution (gassed with 100%  $CO_2$ ) was measured by titration with 5 mM HCl under automatic control from Radiometer pH-stat equipment. The transmembrane electrical potential difference was recorded continuously.

All preparations potently alkalized the luminal side. Subsequent side administration of the prostaglandins  $P_1$  ( $10^{-6}$  M),  $P_2$  ( $10^{-6}$  M),  $P_3$  ( $10^{-6}$  M) or  $P_4$  ( $10^{-6}$  M) increased both the rate of alkalization and the electric potential difference. Concentrations presented are those required for significant (p < 0.05) effect within 60 min after start of exposure. Maximal rate of  $HCO_3^-$  transport was higher with  $P_2$  than with  $P_1$ . The stimulation of surface epithelial  $HCO_3^-$  transport may be one mechanism by which prostaglandins increase the ability of duodenal mucosa to resist acid.

109

Zielland, B. (Department of Biology, Royal Danish School of Pharmacy, Copenhagen, Denmark). **DO THERE EXIST SUBGROUPS OF HISTAMINE  $H_2$  RECEPTORS IN THE GUINEA PIG ILEUM?**

The antihypertensive agent lonididine has been shown to interfere with adrenergic, well histamine  $H_2$  receptors in various tissues. In the present study the possible interference of lonididine with other histamine receptors than the  $H_2$  or  $H_1$  was studied.

The isolated guinea pig ileum conically stimulated by supramaximal rectangular pulse (1 ms duration, 20-40 V, frequency of 1 Hz) was used. Two models were used.

Lonididine concentration-dependently inhibited the twitches of the ileum with an  $IC_{50}$  of 7.0  $\mu$ M. The inhibition by lonididine was concentration-dependently reversed by the  $H_2$ -receptor antagonist burimamid and isetidine.  $IC_{50}$  for reversal was 1.6  $\times 10^{-3}$  and 7.1  $\times 10^{-3}$  M, respectively, whereas metiamide was without effect in concentrations up to 40  $\mu$ M. The  $H_2$ -receptor antagonist also reversed the inhibitory effect of lonididine, with an  $IC_{50}$  of 3.9  $\times 10^{-3}$  M. Substances which interfere with  $H_1$ -receptors (5 HT or opiate receptors) did not reverse the effect of lonididine after blockade of the  $H_2$ -receptor. Histamine itself inhibited the IL-induced twitches, an effect which was reversed by burimamid and isetidine.

The fact that histamine  $H_2$  receptor agonists and antagonists inhibit the same effect on lonididine and in concentrations different from those observed in the isolated tissue may possibly be explained by the existence of histamine receptors in the ileum different from the classical  $H_2$  receptors.



Cassuto J, Jodg M, Lundgren O, and Tettie D  
(Department of Physiol, University of Göteborg  
Sweden): CHOLERA A NERVOUS DISEASE?

The profuse fluid loss in cholera according to current concepts the result of direct stimulation of the cholera toxin on the intestinal epithelium. Some observations, however, indicate that also other mechanisms may be involved. Thus vasoactive intestinal polypeptide (VIP) that is known to be localized in nervous tissues is found in high amounts in the luminal contents during cholera. The cholera exposed intestinal mucosa also exhibit a marked hyperemia, an effect that also may be mediated via nerves (Riber 1973). It was therefore considered of interest to investigate if nervous reflexes may be involved in the pathogenesis of cholera.

The hypothesis was tested in two ways on cats where net water secretion was induced in isolated small intestinal segments by cholera toxin. First addition of the local anesthetic agent lidocaine to the luminal perfusate inhibited the cholera induced secretion and half of the experiments it even produced a net fluid absorption from the lumen. Changing the luminal perfusate back to solution without lidocaine restored the net fluid transport into the lumen. In control experiments lidocaine luminal concentrations that inhibited the net cholera secretion was without effects on the net water uptake from normal intestinal segments. Second, close intraintestinal administration of tetrodotoxin in doses that blocked splanchnic sympathetic nerves also reversibly inhibited the cholera secretion in the lumen.

It is thus proposed that activation of an intramural nervous reflex may be one mechanism of importance in eliciting the fluid loss from the small intestine in cholera. Further, evidence also indicates that VIP may possibly be involved in this nervous reflex.

Reference: Riber J. Acta Physiol Scand 1973 Suppl 401: 1-31

Wroblewski K, Nilsson G, Mikkanen R, and Sundler F  
(Dept of Pharmacology Karolinska Institute Stockholm, Dept of Physiology Faculty of Veterinary Medicine Swedish University of Agricultural Sciences Uppsala and Dept of Pharmacology and Physiology University of London): TIME DEPENDENT INCREASE IN DUODENAL GASTRIN CONCENTRATION IN DOGS FOLLOWING ANTERECTOMY

The main gastrin producing tissue in dogs is the antral portion of the stomach. Basal plasma gastrin level is significantly lowered following antrectomy but markedly reduced after total removal of the duodenal bulb (Nilsson and Wroblewski 1975). Using RIA we have in pilot study demonstrated an increase in duodenal gastrin concentration following antrectomy (Nilsson and Sundler 1977). The time course of this increase was studied in 18 antrectomized dogs where the duodenum was removed 3-10 or 12-18 weeks after surgery. The mean tissue concentration of gastrin in the proximal third of the duodenum increased by time being 73  $\pm$  15, 76  $\pm$  19, 90  $\pm$  20 and 238  $\pm$  67 ng/g tissue respectively compared to 13  $\pm$  8  $\pm$  2 ng/g tissue in unoperated controls. Immunohistochemistry reveals an increased number of gastrin immunoreactive cells in the proximal part of the duodenum in antrectomized dogs. In 3 dogs the mean gastrin concentration in proximal third of the duodenum 8 weeks following total gastrectomy was found to be 48  $\pm$  32 ng/g tissue.

The obtained results confirm the when the main source of gastrin is removed there is a time dependent increase in duodenal gastrin concentration and in the number of G-cells which could be due to feedback mechanism. Although there was no significant difference between the gastrin levels in proximal duodenum after antrectomy and total gastrectomy this does not rule out our previous proposal that the acid producing part of the stomach exerts trophic influence on gastrin production. Since the alteration in acidity and corpus area of food reaching the duodenum following total gastrectomy might stimulate gastrin production and decrease such difference.

Youssef M and Suramo I (Department of Radiology University of Oulu Finland): ULTRASONOGRAPHY IN PHYSIOLOGICAL AND PHARMACOLOGICAL EXAMINATIONS OF THE ABDOMEN

Ultrasonography is a non-invasive technique without any hazards of ionizing radiation. Therefore it is well suited for physiological and pharmacological examinations.

The equipments and techniques have been developed rapidly during the last years. Now it is possible to achieve with gray-scale equipments anatomical information and imaging of the structure of the live pancreas, spleen and kidneys. Real time systems are used to produce dynamic information from vessels, portal and caval veins, aorta and bile duct.

Images of different physiological and pathological conditions are presented.

Kinnula V L, and Hassinen I (Departments of Physiology and Medical Biochemistry University of Oulu Finland): EFFECTS OF HYPOXIA AND FASTING ON THE CYTOCHROME CONCENTRATIONS AND TURN OVER TIME OF INTESTINAL VILLOUS CELL MITOCHONDRIA IN THE RAT

Intestinal epithelial cells probably have the shortest life-span of any mammalian cells which can be harvested quantitatively and therefore suitable for the study of mitochondrial biogenesis. It is provided that their turnover time does not change.

Epithelial villous cells were separated using isotonic mannitol solution and the mitochondria isolated in the same medium. Cytochromes were determined by liquid nitrogen temperature mitochondrial protein turnover was determined by injecting 25  $\mu$ Ci of tritiated leucine intraperitoneally and observing the labelling of mitochondrial protein in up to 120 hrs. Hypoxia was achieved in hypobaric pressure chamber (40  $\pm$  2 kPa).

A paradoxical result in the cytochrome content we obtained is significant increase after 48 hrs in the hypoxic and fasting groups compared with the feeding control. The half-life of the total mitochondrial proteins was 18 hrs in the feeding control and 32 and 36 hrs in the hypoxic and fasting animals. Calculated from the protein in radioactivity during the first 48 hrs.

This increase in cytochrome content is probably due to the prolonged half-life of the mitochondria allowing no extensive synthesis of mitochondrial component. This indicates that the rate of degradation of the mitochondrial component in cell growth may be distorted by change in the life-span of the cell.

114

Ek, B. and Lundgren, B. (Research Laboratories  
A. E. la Mölndal, Sweden) CHARACTERIZATION  
OF  $\beta$ -ADRENOCEPTOR MEDIATED INHIBITION OF SPON-  
TANEOUS CONTRACTILE ACTIVITY IN ISOLATED CAT  
COLON STRIPS

It is known that the spontaneous contractile  
activity in the cat colon strip is inhibited by  
 $\alpha$ - and  $\beta$ -adrenoceptor agonists. In order to  
characterize the  $\beta$ -adrenoceptor mediating the  
inhibition the effect of increasing doses of  
three agonists isoprenaline, prenalterol ( $\beta_1$ -  
selective) and terbutaline ( $\beta_2$ -selective) were  
studied either alone or in combination with the  
antagonist metoprolol ( $\beta_1$ -selective) or IFS 339  
( $\beta_2$ -selective).

The spontaneous activity was characterized by  
recurrent bursts of contractions. The integrated  
contractile activity was totally inhibited by  
isoprenaline ( $PO_2 = 3$ ). The maximal effect pro-  
duced by prenalterol was 36% inhibition ( $PO_2 = 7.3$ )  
and by terbutaline 75% inhibition ( $PO_2 = 7.3$ ).  
The terbutaline effect was blocked by IFS 339  
but not by metoprolol while the prenalterol  
effect was blocked only by metoprolol. The results  
indicate that both  $\beta_1$  and  $\beta_2$ -adrenoceptors  
mediate inhibition of spontaneous activity in  
the cat colon.

The remaining activity after maximal doses  
of prenalterol was often modified into pat-  
tern consisting of irregular contractions of con-  
stant frequency. Some additional experiments  
showed that tetrodotoxin modified the activity  
in a similar way. prenalterol thus the con-  
tractile activity apparently consists of two  
components one dominant neurogenic and sym-  
patico component. We suggest that  $\beta_1$  adrenocep-  
tor stimulation mainly affects the neurogenic  
component and  $\beta_2$  adrenoceptor stimulation the  
sympathetic component.

116

Prithinen, Juhani (Department of Food & Hy-  
giene, Faculty of Agriculture, University of  
Helsinki, Finland) HYPOTONIC  
MORPHOLOGY OF STOMACH DUODENUM AND  
COLON WITH GLUCAGON

Glucagon is a cyclic polypeptide with  
molecular weight of 3485 and  
composed of 29 amino acids and residues.

Glucagon inhibits motility and tone of  
the stomach, small intestine and colon. The  
hypotonic and hypomotility effect is  
comparable to propanteline bromide but  
shorter duration of action. Glucagon  
has practically no side-effect in small  
doses. In dogs, contrast roentgenography  
of stomach, duodenum and colon relaxes  
and inhibits motility. It improves  
the good eating behavior to the stomach.  
Through the effects of glucagon, patients can  
be differentiated from organic changes and  
contrast medium does not pass so early from  
stomach to duodenum. Glucagon is an effec-  
tive and safe drug. Radiographs of stomach,  
duodenum and colon with double contrast  
method using glucagon are reproducible.

115

Puranen, J. (Department of Pharmacology,  
University of Oulu, Finland) GASTRIC MUCOSAL  
BLOOD FLOW IN ETHANOL INDUCED MUCOSAL DAMAGE  
The changes in gastric mucosal blood flow  
induced with ethanol induced damage of the  
gastric mucosa were studied in anaesthetized

rats. The effect of the treatment with 10, 20 and  
30% ethanol in 100 mM HCl pH 1.50 mM NaCl for  
40 min damaged the gastric mucosa. The gastric  
mucosal blood flow was measured by the  
thermo-dilution method. The gastric mucosal  
blood flow was decreased to 50% of the control  
value. The gastric mucosal blood flow was  
restored to the control value by the treatment  
with 10, 20 and 30% ethanol in 100 mM HCl pH 1.50  
mM NaCl for 40 min. The gastric mucosal blood  
flow was restored to the control value by the  
treatment with 10, 20 and 30% ethanol in 100 mM  
HCl pH 1.50 mM NaCl for 40 min.

The type of the treatment with 10, 20 and 30%  
ethanol in 100 mM HCl pH 1.50 mM NaCl for 40  
min did not affect the gastric mucosal blood  
flow. The gastric mucosal blood flow was  
restored to the control value by the treatment  
with 10, 20 and 30% ethanol in 100 mM HCl pH 1.50  
mM NaCl for 40 min. The gastric mucosal blood  
flow was restored to the control value by the  
treatment with 10, 20 and 30% ethanol in 100 mM  
HCl pH 1.50 mM NaCl for 40 min.

The results indicate that the gastric mucosal  
blood flow is decreased by ethanol. The gastric  
mucosal blood flow is restored to the control  
value by the treatment with 10, 20 and 30%  
ethanol in 100 mM HCl pH 1.50 mM NaCl for 40  
min. The gastric mucosal blood flow is restored  
to the control value by the treatment with 10, 20  
and 30% ethanol in 100 mM HCl pH 1.50 mM NaCl  
for 40 min.

117

Seitama, E. (Department of Physiology, University  
of Oulu, Finland) DIETARY HABITS AND FACTORS  
INFLUENCING THEM IN SELECTED FINNISH GROUPS.

A dietary survey among 1175 people in selected  
groups aged 15 to 36 yr was carried out  
using the estimated food records method. To  
study factors influencing food habit question-  
naires were given to 73 of these.

The sporting groups and the farmers ingested  
more foodstuff than others. With the exception  
of these groups the energy intakes were at  
below the level for high physical work. The  
main sources of energy were milk, cereals and  
meat products. Protein, fats and carbohydrates  
covered 12 to 15, 37 to 42 and 48 to 51% of the  
intake energy in the groups respectively.

With the exception of the athletes and ascorbic  
acid, the intake values were parallel to the  
energy intake. The sedentary groups including  
students, servicemen, industrial and office  
workers ingested 1 to 2 times and included  
the greatest proportion (up to 90%) of subjects  
not reaching the recommendation. Iron, vitamin  
A, thiamine, riboflavin and niacin did played  
the least role.

Test health and the price of food and the  
examples as by mother of food and if they had  
the greatest influence on food habits. Nutri-  
tional information came mainly from home  
papers, books and TV. The subjects were  
of recommended practice. The main factors  
affecting habits were the low energy intake  
seen by sedentary life and satisfying the low need  
for energy by food with low nutrient density.  
To improve Finnish eating habits, increased im-  
portance should be given to political decision  
making to help people to encourage  
ed to us more vegetable and non-refined diet.



Larsson, S. Hovell, M. and Holm, A. M. (Department of Physiology and Medical Biophysics Division, Uppsala University, Sweden) **VOLUME, HYDROSTATIC AND OSMOTIC PRESSURES WITHIN RENAL INTERSTITIUM.**

It is well established that tubular fluid reabsorption will be governed by the hydrostatic and osmotic forces operating across the peritubular capillary membrane. The signal will probably be mediated via the renal interstitium. The present paper aims at the study of some tentatively important parameters via the volume and the hydrostatic and osmotic pressure conditions within the renal interstitium.

The interstitial and plasma volumes were measured as the distribution volume of 125-I labelled albumin and 51-Cr labelled EDTA analyzed with a dilution technique with injection into the renal artery and sampling of renal venous blood. Interstitial hydrostatic pressure was obtained from direct punctures of renal subcapsular interstitial fluid. Cell osmotic pressure was analyzed from the protein concentration in subcapsular fluid sampled with small PFC-catheters introduced into the subcapsular space.

During water diuresis conditions the interstitial space amounted to 45 ml/100 g wet increasing slightly 73 ml during 100% extracellular volume expansion. In contrast, the hydrostatic pressure increased steeply from 3.4 mm Hg to 12.1 mm Hg. The corresponding colloid osmotic pressure decreased from 5.2 mm Hg to 1.0 mm Hg during 100% expansion.

The compliance of the interstitium is thus comparatively low. It is suggested that the large interstitial driving forces hampers the removal of the tubular reabsorbates and thereby decreases the peritubular fluid reabsorption from lateral intercapillary space.

Wyllie, D. G. & Liddle, R. W. & J. G. & A. Wyllie, M. (Department of Physiology and Medical Biophysics Division, Uppsala University, Sweden) **INTERSTITIAL CIRCULATION IN ACUTE RENAL FAILURE.**

Acute renal failure was induced by 45 min clamping of the renal artery. This produced classical acute renal failure with very small filtration, anisotermic polyuria and reduced potassium secretion. Microspectroscopy showed that the reduced tubular filtration was caused by either obstruction of the tubular lumen probably located in the medullary region or by glomerular ischemia, with subsequent collapse of the nephron. The renal arterial blood flow analyzed with the microsphere technique or the 86-Rb extraction method showed a 60% decrease of the total renal blood flow. The medullary blood flow was under normal conditions, 2.5 l/min and 5 g/min in the outer medulla decreasing successively towards 1.0 ml/min in the inner medulla. During renal failure the blood flow in the inner cortex and the outer parts of the inner medulla was decreased 100% and 100% of the normal blood flow. This ischemic medullary blood perfusion caused an impaired function of the medullary cellular segments resulting in deficient urine concentration ability. Microscopic examination of kidney tissue revealed congestion and stagnant blood in the vessels with reduced blood flow predominantly in the inner cortex of the outer zone of the medulla.

Nilsson, A., Odling, B. and Nilsson, L. (Department of Medical Pharmacology and Medicine, University of Uppsala, Sweden) **PERITUBULAR EFFECTS OF INHIBITORS OF THE AVIAN KIDNEY.**

In an attempt to study the renal peritubular space,  $\beta$ -proteins were injected 125 I labelled porphyrin inulin human  $\beta_2$ -microglobulin ( $\beta_2$ -m) and  $\beta$ -microglobulin ( $\beta$ -m) together with 51-Cr-EDTA into the renal portal system of the kidney (10-15  $\mu$ l per protein) prepared according to the modified technique technique. The 125 I to 51-Cr activity ratio in the injection mixture was approximately 1. The animals were killed 1, 4 or 18 (30) min post-injection and the ipsilateral (injected side) and contralateral (control side) kidneys and the organs were removed. The validity of the method was verified by calculating the corresponding 125-I to 51-Cr EDTA activity ratios after injection of 125 I  $\beta$ -m or  $\beta$ -m. The maximal  $t_{1/2}$  (15.6  $\pm$  0.4 s.d.) was found 1 min in the ipsilateral kidney. The corresponding values for the control kidney were 2.7  $\pm$  0.8 s. A ratio below 1 was found in all other organs. At 1 min there was high retention in the ipsilateral kidney (4.4  $\pm$  0.2 s.d.) significantly greater than in the control kidney (1.9  $\pm$  0.2); at 10 min the ratio was about 2 and approximately equal in the two kidneys. For  $\beta$ -m and  $\beta$ -m there was no increase in the retention for either kidneys compared to the injection solution. This was true also for  $\beta_2$ -m at 1 min while the ratios at 10 and 30 min were around 2, equal for the two kidneys. **Conclusion.** The method used appears valid for studying renal peritubular uptake of renal anions. There seems to be a significant peritubular uptake in the avian kidney.  $\beta$ -inulin but not the other proteins studied.

Odling, B. (Department of Medical Pharmacology, BMC Unit, Uppsala University, Sweden) **RENAL TUBULAR SECRETION AND EFFECTS OF A NEW OSMOTIC DIURETIC (HOE 740), AN ALKALINE SULPHONAMIDE.**

The modified Sparer technique was used to study the renal tubular secretion of tubular secretion and salivary secretion of 14-C timololol (10-15  $\mu$ g/kg/mi) before and during co-administration of 100  $\mu$ g/kg of HOE 740 (novobiocin) 1.7  $\mu$ g/kg/mi or cat on (Dantrolene -methylsulphonate)-0.1 25  $\mu$ g/kg/mi) transport T and inhibitors were injected into the renal portal system of the kidney. The renal clearance of 14-C-EDTA ( $C_{EDTA}$ ) and 125-I- $\beta$ -m ( $C_{\beta}$ ) were determined. **Result.** The tubular secretion efficiency of T was markedly reduced (to 5-32% of pre-injection values) by Dantrolene, but it was unaltered by novobiocin. The effect on T was to one level, resulting in an equal renal excess secretion of sodium of 45.2  $\pm$  17.7% (5.0% of total sodium) and percentage of the total sodium secretion. A corresponding value was obtained for the excess secretion of chloride which that of potassium was small (3.3  $\pm$  0.5%). Dantrolene reduced these excess secretions of sodium and chloride roughly in proportion to the reduced TEF values of T while they were only slightly (3-4% for sodium about 10%) reduced by novobiocin. T had no effect on  $C_{EDTA}$  or  $C_{\beta}$ . Novobiocin reduced  $C_{\beta}$  markedly (to 10-15% of pre-injection values) (to about 1/4 and 3/10 of pre-injection values resp.) while these were unaffected by Dantrolene. Conclusion 2. The Sparer preparation T is actively secreted into the urine by organic cation transport. The secretory effect of T seems to be separate from its natriuretic and diuretic effects; it transports T respect T differs from acid sulphonicamide diuretics (this idea) but resembles loop-diuretics. Thus, significant part of the salivary effect of T is then appears to be evolved from the luminal side of the nephron.

Hansen O, Larsen J A and Thomsen O #  
(Institute of Physiology University of Aarhus  
Denmark) BIOLOGICAL ASPECTS OF VANADATE INTER-  
ACTION WITH THE SODIUM PUMP

Since the discovery of vanadate transport inhibitors of NaK ATPase considerable interest has been focused on its possible biological importance. Vanadate is chemically related to phosphorus.

In the present experiments some characteristics of vanadate-ATPase interaction were studied. The specificity of  $(^{45}\text{V})$ -binding was demonstrated by (1) its facilitation by  $\text{Mg}^{2+}$  K (K<sub>0.5</sub> 2-4 mM and its inhibition by  $\text{Na}^{+}$  ATP and PI and by (2) the identity of the total number of vanadate nucleotide and ouabain binding sites in activated ATPase preparations from ox brain, pig kidney and rat liver and dogfish renal gland. A method was developed for determination of vanadate in serum by measuring the displacement of bound  $(^{45}\text{V})$ vanadate to NaK ATPase with unlabeled vanadate.

Vanadate has been shown to act as a potent diuretic and natriuretic agent. In the rat we infused vanadate in anesthetized cat and were unable to reproduce these effects whereas in a blood pressure 74% decrease in renal blood flow and secondary 90-100% reduction in urine flow was observed.

It appears that in the rat vanadate acts as a potent vasoconstrictor. Vasoconstriction was evoked by serum vanadate concentrations of about 30  $\mu\text{M}$  as determined by the above-mentioned method.

(1) Balfour W E # 1 (1978) Nature 275 768.

Persson B E and Persson E (Department of Physiology & Medical Biophysics University of Uppsala, Sweden)  
MODULATION OF GLOMERULAR FILTRATION RATE VIA PLASMA PROTEIN CONCENTRATION

In polythionamide-like Amphibian means the urinary excretion rate changes on alteration of the extracellular volume to a large extent by means of changes in GFR. In an attempt to determine whether this effect is mediated by changes in the colloid osmotic pressure of the blood, experiments were performed in which an amphibian Ringer solution with or without 3g % bovine albumin was perfused into arteries supplying blood to 2-5 glomeruli. On the kidney surface arterial supplying blood to several glomeruli are accessible for micropuncture. The perfusion rate was about 30 nl/min. Aspiration. At the same time a pressure recording cannula connected to a servo-nulling pressure-recording system was used to puncture the afferent arteriole immediately before its division into the glomerular capillary network (GCP). It was found that on perfusion of the artery with the solution without albumin GCP increased significantly ( $p < 0.001$ ) from a control value  $1.9 \pm 0.9$  to  $14.3 \pm 8.8$  cm H<sub>2</sub>O (n=15). When amphibian Ringer solution containing 3g % albumin was used GCP decreased from  $9.4 \pm 0.5$  to  $4.5 \pm 0.8$  cm H<sub>2</sub>O (n=7). From these results it can be postulated that changes in colloid concentration might on their own by which slight glomerular blood flow, GCP and urinary excretion are altered in response to changes in the degree of hydration.

Ensmen K E (Institute of Medical & Biophysics University of Aarhus, Denmark)  
GLOMERULAR FILTRABILITY OF FLEXIBLE  
POLYMERS IN THE RAT

The excretion of different polyethylene glycol and dextran solutions with narrow molecular weight ranges was compared to that of inulin in the anaesthetized rat. The clearance of polyethylene glycol with molecular weight up to 4000 was identical to that of inulin. The molecular weight distribution of inulin in rat plasma and urine was identical indicating no restriction to ultrafiltration of inulin in the glomerular filtration barrier. Dextran declined from 100% towards 0% as Stokes radius  $r_s$  increased from 18 Å to 46 Å. The decline of ultrafiltrability of polyethylene glycol occurred at lower  $r_s$  values than for dextran. Furthermore dextran and Sephadex G-200 gel to a higher extent than do polyethylene glycol of the same Stokes radius. The dependence of Stokes radius and intrinsic viscosity on molecular weight indicated that polyethylene glycol is more expanded in aqueous solution than is dextran. This difference is attributed to chain branching of the latter polymer. It is concluded that flexible polymers probably because of steric hindrance permeate through water-filled pores to a lesser extent than compact molecules of similar Stokes radius. Assuming that the apparent molecular radius of a polymer is proportional to the square root of its molecular weight, it is concluded that the apparent molecular radius of a polymer is determined by its penetration into Sephadex G-200 columns calibrated with globular proteins. The glomerular sieving data of the present study suggest an effective channel half-width for the glomerular sieving of 35 Å.

Marild, J. and Sheikh, M I. (Institute of Medical Biophysics University of Aarhus, Denmark) ROLE OF  $\text{Na}^{+}$  ON ACTIVE SODIUM TRANSPORT OF PAN

The relation between metabolic oxygenation of renal PAN accumulation and  $\text{Na}^{+}$  transport has been studied in rabbit cortical slices in the presence of various metabolic inhibitors and under anaerobic conditions. 2,4-dinitrophenol (DNP) and  $\text{F}^{+}$  at low medium concentrations giving rise to a light decline in oxygen consumption and tissue ATP concentration caused reduction of PAN accumulation without significantly affecting intracellular  $\text{Na}^{+}$  concentrations. Inhibition of PAN accumulation under these conditions could be counteracted by addition of metabolic substrates. Observations in the presence of lipomylin suggest that this compound causes a moderate reduction of both PAN accumulation and  $\text{Na}^{+}$  transport. Studies on the effect of oxygen-mediated PAN uptake showed that transport slows under anaerobic conditions relative to that measured under aerobic conditions in  $\text{Na}^{+}$ -depleted slices. In the latter case the maximal accumulation time achieved was 1.35  $\pm$  0.16 supposing that  $\text{Na}^{+}$  is indispensable for the attainment of high accumulation time. The uptake of PAN under anaerobic conditions could be inhibited by probenecid (1 mM) and high concentrations of 2,4-dinitrophenol (5 mM). However, the concentrations of 2,4-dinitrophenol and  $\text{F}^{+}$  used for metabolic inhibition under aerobic conditions did not significantly affect PAN transport in the absence of  $\text{O}_2$ . It is concluded that both  $\text{Na}^{+}$ -dependent and  $\text{Na}^{+}$ -independent component exists in the energetically coupling of metabolism to active PAN transport.

130

Bealch, M.I. and Maxild, J. (Institut f Medical Biochemistry University of Aarhus Denmark) KIDNEY HANDLING OF PROBENECID AND ANALOGUES

The characteristics of renal transport of homologous series of hydrocarbon derivatives of sulfonyl benzoic acid were examined in cortical slices of rabbit kidney. The length of the hydrocarbon substituent varied from  $C_1$  to  $C_8$  and the compounds differed from the previously examined probenecid series in having only one hydrocarbon substituent instead of two, the sulfonyl group. Transport rate, maximal accumulation and affinity of the monosubstituted compounds for transport were enhanced by an increase in the length of the hydrocarbon substituent. In comparison with the probenecid series, less binding by tissue constituents under anaerobic conditions was observed. Competition experiments indicated that the monosubstituted compounds were transported by the same transport system as that of PAM. Oocyte assays and transport started biphasic effect on accumulation (i.e. stimulation at low and inhibition at high metabolite concentrations) as in the case of other organic anions. In comparison with probenecid, the monosubstituted compounds were more susceptible to inhibition by PAM, furosemide and ouabain. The monosubstituted compounds were less hydrophobic than probenecid as evidenced by less extractability from an aqueous phase into an organic liquid (1,2-dichloroethane). It is concluded that for the sulfonyl benzoic acid derivatives higher hydrophobicity results not only in higher affinity but also in an enhanced turnover rate of the carrier system for organic anions.

132

Wettersen, M.W. and Eriksson, L. (Department of Physiology College of Veterinary Medicine and Department of Veterinary Medicine University of Helsinki Finland) RETENTION OF ELECTROLYTES AND WATER DURING THE MATING SEASON IN REINDEER.

Seasonal environmental factors do not completely explain the annual cyclic alterations of water and mineral balance in reindeer. In this study, periodic changes in urinary sodium and potassium excretion were followed in reindeer during three years. The animals were kept on low, high protein diets with and without daily supplement of 10 g NaCl.

On both high and low protein diets with and without NaCl supplement, significant decreases in sodium excretion occurred during the mating season. Concurrently, there was a decrease in potassium excretion when the animals were on high protein diet with its inherently high potassium content. However, the plasma sodium and potassium concentrations did not change. On low protein diet without NaCl, the urinary water excretion was significantly lower than in animals on high protein diet. There were no differences in the response of electrolyte excretion to NaCl supplement between the two groups. It is likely that the mating season is associated with a decrease in sodium and potassium excretion.

131

Kopp, U., Aurell, M., Svensson, L. and Ahlstedt, B. (Dept. of Pharmacology AB Hassle Mölndal, Sweden and Sahlgrenska Hospital Göteborg, Sweden) EFFECTS OF PRENATAL AND POSTNATAL ADRENORECEPTOR AGONIST ON RENAL FUNCTION IN ANAESTHETIZED DOGS

Prenatal treatment with the selective  $\beta_1$ -adrenoreceptor agonist (Carleson, B. & M. Nauyn, Scheideberg Arch Pharmacol 300:101, 1977). We have studied the effects of prenatal treatment on kidney function in order to explore whether  $\beta_1$ -adrenoreceptors are involved in the control of renal release, renal blood flow and secretory functions. Result: Prenatal treatment with 30  $\mu$ g/kg increased heart rate by 40 beats/min without changing mean arterial pressure. Prenatal treatment increased renin release by 760% ( $p < 0.05$ ) the effect being independent of an intact renal innervation.

Prenatal treatment tended to increase renal blood flow. The renal vasodilator effect was more marked in dogs undergoing water diuresis where prenatal treatment reduced renal vascular resistance by 14% ( $p < 0.05$ ) in both innervated and denervated kidneys.

Postnatal treatment with metoprolol 5 mg/kg  $\beta_1$ -adrenoreceptor antagonist reduced the chronotropic effect of prenatal treatment and abolished the effects on renin release and renal blood flow.

While glomerular filtration rate and sodium excretion were unaffected by prenatal treatment, reduced urine volume (30-30%) and an increased urine osmolality (60%) was produced by the drug. The latter effects were prevented by vagotomy and therefore probably due to an increased release of ADH. Reflex response secondary to the cardiostimulant effect of prenatal treatment of the renal vasculature.

Conclusion: The results support the existence of  $\beta_1$ -adrenoreceptors mediating renin release and dilatation of renal resistance vessels.

133

Hanninen, M., Koskela, M., Leinonen, M. and Vartiainen, J. (Departments of Ecology, Medical Microbiology and Physiology University of Oulu Finland) GROWTH AND SERUM PROTEINS IN THE REINDEER.

The reindeer in Finland is grazing in the forests and subarctic mountain areas undergoing an annual physiological regimen with highest metabolic demands occurring in the spring and early summer. So it has been studied the age and sex related growth and electro- and immunoelectrophoretic serum protein changes in 167 reindeer. The rapid weight gain is maximal (400 g/day) at an age of 4 to 8 weeks and the first winter stops the growth and so the growth can be depicted by 2 separate polynomial functions.

About 15 protein bands are discernible in the serum showing wider distribution in alpha region on IgG and also higher cathodal mobility of IgA than in human serum. The total serum protein is low in the age of 20 days but high in the adult hind and the difference is caused by the protein changes. The mating season decreases the protein levels of stages as compared with those of hinds. The gammaglobulin is high in the newborn calf but low 3 weeks later and highest in the adult hind.

It is suggested that the reindeer calf acquires passive immunity soon after the birth by intestinal absorption of immunoglobulins and also that the endogenous immunoglobulin synthesis begins in the fourth week of life and hence the period just prior to this forms a critical stage partly explaining the high mortality of the reindeer calf during the strenuous summer brought by drought and blood-sucking insects.

## 142

Bredin, K. Oleson & Olgart L. and Nilsson, G. Dept. of Pharmacology, Karolinska Institute, Stockholm and Dept. of Physiology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala

# STIMULANT P-LIKE IMMUNOREACTIVITY (SPLI) IN THE DENTAL PULP TISSUE CONCENTRATION, RELEASE AND DEGRADATION

The concentration of SPLI in dental pulp from cat dog and man determined by RIA was found to be comparable or higher than levels found in other tissues outside the CNS. The chromatographic behavior of SPLI on Sephadex G 25 in pulp extract from cat was similar to that of synthetic bovine SP. Incubation of cat canine teeth at 37°C for up to 4 hrs did not influence pulp SPLI content compared to levels in contralateral unexposed teeth. Similar incubation of homogenized pulp tissue led to rapid decreases in SPLI concentration in the homogenate. Synthetic bovine SP and  $\beta$ -tyr-SP were

rapidly destroyed by homogenized pulp tissue. Release of an idiosyncratic form of the ipsilateral mandibular nerve (10V 15 Hz 3ms) prior to the incubation of the intact teeth led to reduction of the SPLI content in pulp on the stimulated side to 50-60% of the levels found in the contralateral control teeth pulp. The same type of nerve stimuli also increased the concentration of SPLI in pulp peptidase fractions (20-42 pg/50  $\mu$ l compared to 0-10 pg/50  $\mu$ l during control periods) and caused a rapid increase in pulp blood flow by 73% that was not blocked by procaineol (0.5 mg/kg), trypsin (3 mg/kg), neoprene (3 mg/kg) or imidazole (3 mg/kg).

The demonstrated release of SPLI and increase in pulp blood flow following nerve stimulation together with potent vasodilator action of SP in this organ indicate that SP may contribute to the development of inflammatory responses associated with injury and pain in peripheral organs.

## 143

Jarvik, A., Lindberg, J. H., Kivik, T., Nilsson, G., Fahlström, J. and Öst, B. (Dept. of Oto-rhino-laryngology and Otolaryngology, Karolinska Institute, Stockholm, Dept. of Physiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, Dept. of Clinical Chemistry, Huddinge Hospital, University of Copenhagen, Denmark and Dept. of Internal Medicine, Dallas, Texas, USA)

# IMMUNIZATION OF CAT NASAL MUCOSA WITH SPECIAL REFERENCE TO RELATIONS BETWEEN PEPTIDOMIMIC AND CHOLINERGIC STIMULI

The origin and distribution of nerves of rat nasal mucosa in normal and desensitized animals were studied by immunohistochemistry using antibodies to substance P (SP) and VIP combined with staining for acetylcholinesterase (AChE). SP positive (11) bodies were found in the trigeminal ganglia, while dense network of terminal-like structures were seen in the sphenopalatine ganglia (SG) around the principal ganglion cells. In the nasal mucosa thin SP immunoreactive nerve fibers were seen in the subepithelial layer sometimes penetrating into the epithelium. VIP immunoreactivity was observed in the majority of SG cells and in dense networks of nerve terminals in the nasal mucosa surrounding glands and blood vessels (mainly arterioles but also some venules). AChE staining of the same section revealed very close overlap between AChE-rich and VIP positive cells in the SG. Furthermore the distributions of AChE and VIP in the nasal mucosa were similar. After activation of the SG almost all VIP positive and AChE stained nerves disappeared in the nasal mucosa. The present data thus suggest peristaltic presence of VIP in some presumably cholinergic neurons (AChE rich) of cat innervating exocrine glands and blood vessels. (See also Lindberg, J. H. this volume.) The use of AChE as marker for cholinergic neurons should however be interpreted with caution. On the basis of the present findings, hypohidrosis is advanced that local reflexes exist involving sensory SP nerve endings in the nasal mucosa and in the sphenopalatine ganglion and vasomotor secretory (VIP-cholinergic) neurons.

## 144

Koivumäki, J., Riihimäki, M., Leppälampi, J. and Saikku, A. (Department of Physiology, Faculty of Medicine and Anatomy, University of Oulu, Finland)

# PANCREAS AS A SOURCE OF TRYPTOPHAN-RELEASING-FACTOR (TRF)

We have previously shown that methanolic extracts of rat gastrointestinal tissues contain immunoreactive TRF which is cation exchange and high-pressure liquid chromatography behavior is synthetic TRF. The highest concentration was found in the pancreas and this peptide deals with the release of pancreatic TRF.

Rat pancreas slices were isolated after collagenase hydrolysis and submitted with the exocrine part to cation exchange chromatography. The fraction corresponding to the elution of synthetic TRF was assayed by TRF radioimmunoassay. The eluate contained 0.211  $\pm$  0.07 and the exocrine part 0.01-0.01 pg of TRF per  $\mu$ g of protein.

Trifluoromethylated TRF injected in mice and whole body autoradiography were found that the pancreas was uniformly labeled. The synthetic TRF was injected into mice no accumulation of immunoreactive TRF in the pancreas could be found. Our preliminary immunohistochemical studies show that the TRF-like material is localized in the outer part of the islet of Langerhans in mice with an  $\alpha$ 1 TRF serum.

At the moment it seems that the pancreas is a source of TRF. The release of TRF

## 145

Selkälä, H., Toomari, M. and Leppälampi, J. (Department of Physiology, University of Oulu, Finland)

# HIGH-PRESSURE LIQUID CHROMATOGRAPHIC EVIDENCE OF THE PRESENCE OF LRF IN RAT PANCREAS

Several biologically active peptides are known to be located in neuroendocrinally originating tissues. For example, hypothalamic hypothalamic peptides somatostatin and TRF have been shown to occur in gastrointestinal tissues. In this report we show that LRF, hypothalamic hypothalamic peptide hormone, is also located in gastrointestinal tissues.

Rat gastrointestinal tissues were extracted with methanol (9 parts). The evaporated residue was submitted to CM-chromatography in 18% acetic acid followed by 200 mM ammonium acetate corresponding to the elution of synthetic LRF were lyophilized and assayed by LRF radioimmunoassay. Pancreatic extracts showed the highest LRF level, 1 pg/mg. The fractions were then submitted to HPLC (MCN-column) with increasing acetonitrile gradient. Pancreatic LRF immunoreactivity was retained at 11-14 min, the same time as synthetic trifluoromethylated LRF. Our immunohistochemical studies (to be published) showed that pancreas tissue contained specific LRF positive material. Hence we infer that LRF also belongs to the biologically active peptides common to brain and intestine.





Pöytälahti M. and Saarala S. (Department of Zoology, Zoophysiology Laboratory, University of Oulu, Finland) EFFECTS OF BLOOD PRESSURE MANIPULATIONS ON SHIVERING IN THE PIGEON

Although often included in a model of temperature regulation, non-thermal influence on thermoregulatory effectors have received little experimental interest. We have studied changes in shivering induced by blood pressure manipulations in the pigeon.

Blood pressure (BP) from brachial artery and shivering (integrated pectoral EMG) were measured from conscious pigeons placed in metabolic chamber at 10°C. Noradrenaline (NA) was used to raise and sodium nitroprusside (SNP) to reduce BP, respectively. An i.m. i.v. injection of NA inhibited shivering when the increase in BP reached about 45 mmHg. The inhibition had dynamic nature being more complete when BP changed rapidly. Shivering was later restored when BP started to decline. When BP was normalized by i.m. or i.v. injection of SNP, a strong burst of shivering usually followed. However, reduction of BP below normal had potent inhibitory effect on shivering, which was not abolished until BP started to rise again.

The results indicate that blood pressure in other physiological regulatory variables may have profound effects on the thermoregulatory control system. This suggests a hierarchical relation between body temperature control and other homeostatic mechanisms.

Lahti K. and Pyörriä R. (Department of Zoology, Zoophysiology Laboratory, University of Oulu, Finland) THERMOREGULATORY EFFECTS OF CARBACHOL IN THE POSTERIOR HYPOTHALAMUS IN THE PIGEON

Thermoregulatory responses to intrahypothalamic injection of carbachol (CCh) were recorded from unanesthetized pigeons exposed to 20°C and 38°C. The drug (0.25-1.0 µg/µl) was administered to the posterior hypothalamus, which seems to be principally concerned with the control of the response to ambient cold.

At 20°C CCh in most cases either produced cessation of shivering and led to hypothermia or produced biphasic response. A light increase in body temperature was followed by hypothermia. In one pigeon the injections intensified shivering and an increase in body temperature was seen. The responses to CCh were not clearly dose-dependent.

Under moderate heat load (38°C) CCh depressed the thermally induced peaking and caused hyperthermia. A light increase in heart rate was also recorded. Not even these effects of CCh were dose-dependent.

The responses to CCh in the posterior hypothalamus were not as unambiguous as in the anterior/medial parts (Pyörriä & Lahti, Neuropharmacology 16: 737-741, 1977; Neuropharmacology 1979, in press). The results suggest, however, that cholinergic mechanisms located in the posterior hypothalamus are involved in temperature regulation in the pigeon. Evidence for the existence of a pathway mediating locomotor heat gain was obtained, but the exact site at which CCh produces different responses remains to be elucidated.

Li A. (Department of Zoology, Zoophysiology Laboratory, University of Oulu, Finland) FAILURE OF PALAESTRAL LESIONS TO AFFECT THE APOMORPHINE-INDUCED HYPOTHERMIA IN THE PIGEON

Apomorphine (intravenously or intracerebrally) evokes hypothermia and certain effects in the pigeon. The precise location of central dopaminergic receptors which mediate these effects seems to be uncertain. Since dopamine is found in great quantities in the paleostriatum in the pigeon brain, experiments were carried out to determine if this part of the brain is involved in temperature effects of apomorphine.

Eight birds were tested first. The experiments consisted of placing an unanesthetized, trained pigeon in the metabolic chamber (ambient temperature 20°C) and injecting apomorphine (5 mg/kg i.v.) shortly after about 2 h adaptation to the temperature. The maximum change (ΔT) in body temperature was 1.8 ± 0.2°C (n=6). 60-70 min after injection, bilateral electrolytic lesions (2 mA anodal current, 20 sec) of the paleostriatum were made. The body temperature fall induced by apomorphine (5 mg/kg i.v.) 2-4 days after surgery was 1.5 ± 0.2°C (n=6). The difference in temperature between the lesioned bird and the control bird was not significant (p > 0.05, t-test).

A model theory in temperature regulation of the mouse has been postulated of dopaminergic neurons of the subpretectal nucleus (caudate) corresponding to the avian paleostriatum. The present results suggest that paleostriatum does not contribute to apomorphine hypothermia in the pigeon. Supported by grant from the Finnish and Nilsen Research Foundation.

Uchijima, M. (Institute of Physiology, University of Aarhus, Denmark) A VOLTAGE-CLAMP ANALYSIS OF THE EFFECT OF TETRACALIN ON AN IDENTIFIED SMALL NEURONE

After impalement of an identified *Helix aspersa* neurone (cell A, Meech & Physiol. J. 249 (1975) 239) with two independent, exposed microelectrodes, voltage-clamp studies were made using conventional techniques. During depolarizing step the cell displayed fast inward current and delayed outward current. Ion substitution experiments showed that about 1/3 of the fast inward current is carried by Na<sup>+</sup> (I<sub>Na</sub>) and 2/3 by Ca<sup>2+</sup> (I<sub>Ca</sub>). The delayed outward current is carried by K<sup>+</sup> and has two components: one is voltage dependent (I<sub>K(v)</sub>) and the other is activated by Ca<sup>2+</sup> influx (I<sub>K(Ca)</sub>). Superfusion with 0.1 mM tetracalin inhibited I<sub>Ca</sub> (by 25%) more than I<sub>Na</sub> (5%), 0.5 mM tetracalin inhibited I<sub>K(Ca)</sub> (75%) more than I<sub>K(v)</sub> (40%).

Tetracalin blocks Na<sup>+</sup>-channel in many other tissues at much smaller concentrations than used here (e.g. Skou, Acta Pharm. 10 (1951) 291). In this preparation, 11 local channels investigated were inhibited by tetracalin but displayed different sensitivities. Thus the inhibition is not merely an unpecific effect.

If the relatively high concentrations used

Jensen-Bjerg, J. (Department of Biophysics University of Copenhagen Denmark) CHOLINESTER DETECTION BASED ON AN IODINE IODIDE PRECIPITATION (IODINE-STARCH REACTION; TRACER TECHNIQUE; ULTRASTRUCTURAL LOCALIZATION)

Acetylcholine is precipitated by treatment with a strong solution of sodium polyiodide in water ( $\text{NaI } 1, - \text{NaI } 1$  where  $n$  is average molecular weight  $10^6$ ). One molecule of the ester is precipitated together with 8-10 atoms of iodine. A blue colour is developed with soluble starch and read at 575 nm. The molar extinction is up to 80 000 and less than 1000 can be determined. By use of  $\text{NaI } 1$  the sensitivity markedly increased (counting per formed in an Auto-Gamma spectrometer).

For electron microscopy the fixed and polyiodide treated specimens are washed with ether followed by an ether-Epon series and finally embedded in Epon. The presumed cholinester-containing vesicles are heavily stained with an electron dense precipitate (acetylcholine-polyiodide).

Morgberg, A. and Sundvall, A. (Dept of Pharmacol. Univ. of Uppsala and Dept. of Clin Pharmacol. Karolinska Institute Huddinge Hospital Sweden) ASSESSMENT OF REGIONAL CHOLINERGIC NEURONAL ACTIVITY

Endogenous acetylcholine (ACh) ACh-turnover and high affinity choline uptake (H.A. Ch uptake) have all been considered valuable markers for cholinergic neuronal activity. The present communication compares the markers regarding regional differences in the brain of normal mice as well as in mice treated with oxotremorine (OT) and sodium pentobarbital. A relationship is obtained between *in vivo* turnover values for five different brain regions and  $V_{max}$  values for *in vitro* H.A. Ch uptake.

Pre-treatment with OT and sodium pentobarbital produces specific regional changes in both turnover and H.A. Ch uptake. However, both drugs produce shifts of the correlation lines due to uptake being less affected than turnover. Atropine completely prevented the effects of OT regarding turnover and H.A. Ch uptake.

Separation of Ch metabolites in the synaptosomes revealed differences between the brain regions but the drug treatments had only minor effects on the proportion of  $^3\text{H}$ -ACh formed. This is a major difference from what is obtained when Ch is given by intravenous injection when both drugs produce an extremely marked decrease in the  $^3\text{H}$ -ACh formation. Turnover thus seems more sensitive marker for cholinergic activity than H.A. Ch uptake. The combination of the two methods has indicated dissociation between uptake of Ch and synthesis of ACh.

Steen, A., Jørgensen, and Henningsen, D. (Inst. of Medical Physiology, Dept. A, the Danish Institute of Neurophysiology, Copenhagen, Denmark) INFLUENCE OF HYPOXIA ON KIPPOONAL NERVE CELLS IN VITRO

The extracellular concentration of  $\text{K}^+$  in brain increases rapidly during cerebral ischemia (Steen, A. and Jørgensen, D. 1978, 103, 134) whereas  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations all (Steen, A. unpublished). These shifts between intra- and extracellular space may be due to inhibition of active transport or increased membrane permeability or both.

Kippoonal slices from decapate pig were equilibrated in Ringer perfused with Ringer solution equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The input resistance of intracellularly recorded CA1 pyramidal cells was measured. The cells were synaptically activated by stimulating the Schaffer collateral and the minimal stimulus strength necessary to evoke action potentials was determined. Hypoxia was produced by perfusing the chamber with Ringer perfused with 95%  $\text{N}_2$  and 5%  $\text{CO}_2$ . The oxygen tension of the chamber medium was monitored.

In response to hypoxia the input resistance decreased and the threshold of generating action potentials increased until no action potentials could be evoked. Decreased until no action potentials could be evoked. During these changes the cells initially hyperpolarized 5-10 mV and then depolarized. Upon reperfusion the cells returned to Ringer so when the cells were repolarized. Intracellular recordings could not be maintained. Other cells in the same slice isolated after hypoxia had normal membrane potential input resistance and three action potentials.

This study demonstrates that hypoxia induces decreases of input resistance. The initial fast decrease of input resistance is mainly due to an increase of  $\text{K}^+$  conductance as shown by the hyperpolarization and the slow decrease of input resistance is mainly due to an increase of  $\text{Na}^+$  conductance as shown by the depolarization. The rapid shift observed in input resistance can be explained by increased membrane permeability.

Olsson, K.A. and Lundgren, S. (Dept of Physiology, University of Lund, Sweden) FACILITATION AND INHIBITION OF JAW REFLEXES EVOKED BY ELECTRICAL STIMULATION OF THE CEREBRAL CORTIX IN THE CAT

The effects of conditioning electrical stimulation (1-10 msec pulses 0.5 ms duration 400-600 Hz) of the cerebral cortex on the mono-synaptic jaw closing and the di-synaptic jaw opening reflexes were studied in cats anaesthetized with chloralose.

Alternating phases of facilitation and inhibition of the reflexes were observed. The reflexes were 1) fully inhibited or facilitated with 1 stimulus as short as 3-5 ms which may indicate di- or tri-synaptic paths from the cerebral cortex to the trigeminal motoneurons.

The most efficient effects originated 1) from the coronal gyrus. Large effects were evoked from area 3b whereas areas 4, 5a, 5b and 42 were less efficient. The observations indicate that the 1) projection from the jaw reflexes were evoked from the primary projection from the oral cavity and the face (the projections of the 1) spinal tract, 2) spinal nerves and the nerve to the contralateral maxillary whisker).

Hansen, A. (Institute of Medical Physiology Dept A, The Panum Institute Copenhagen Denmark) **BRAIN EXTRACELLULAR IONS IN ISCHEMIA AND SPREADING DEPRESSION**

The extracellular concentration of potassium  $K_0$  in brain increases in ischemia and spreading depression (SD) (Gjessens A.J. Acta Physiol Scand, 1977 92: 412) is unknown however to what extent the concentration of other ions are affected

Spontaneously breathing rats were anesthetized with pentobarbital and bur holes placed in the parietal and frontal bones. Microelectrodes sensitive to  $K^+$ ,  $Na^+$ ,  $Ca^{++}$  and  $H^+$  were inserted in the extracellular space of parietal cortex pH was measured with glass electrode while other ion concentrations were measured with liquid-ion exchange electrodes. SD was elicited by briefly stimulating the frontal cortex with needle and was monitored by the transient negative change of the DC-potential. Following the passage of SD ion concentrations were allowed to recover before the heart was arrested by injecting saturated  $KCl$ .

Normal ion concentrations were ( $mm$ )  $K_0$  3.1  $Na_0$  153  $Ca_0$  1.0  $Cl_0$  1.1 and pH 7.4. During SD the concentrations transiently changed to ( $mm$ )  $K_0$  50  $Na_0$  38  $Ca_0$  0.05 and pH 7.2. The variations of  $Ca_0$  and pH were of longer duration than the changes of the other ions. At the onset of ischemia pH immediately began to fall as  $K_0$  slowly increased while the other ions did not change significantly. After 12 min of ischemia when the DC-potential became negative  $K_0$  increased rapidly and  $Na_0$ ,  $Ca_0$  and  $Cl_0$  showed sudden decrease. Extreme loss of bar 12 min of ischemia were ( $mm$ )  $K_0$  80  $Na_0$  44  $Cl_0$  0.05 and pH 7.0.

These brain cells are unable to maintain ion gradient the onset of SD and after few min of ischemia.

Ahl, L.M.J. I. h. on R. ad Ah. l. (Di. l. l. f Ph. m. l. ay. Dep. m. l. Ph. m. y. U. l. i. y. f M. l. i. h. l. 74. l. ad.) **EFFECTS OF ACUTE MORPHINE ADMINISTRATION ON CATECHOLAMINES IN DIFFERENT PARTS OF THE RAT BRAIN**

Th. ff. f morph. l. the. f. f. m. hyl. p-yr. f. i. (MT. 100 mg/kg. i. p. 3 h.)  
 i. d. m. b. l. d. p. m. i. (DA) d. i. d. a. a. l. i. (NA) depl. t. ion. m. d. i. d. m. l. v. i. t. d. z. d. i. g. l. i. d. m. d. the morph. d. m. q. i. d. Morph. d. m. i. t. d. d. t. diff. t. i. g. l. d. (K. p. A. 10 g/kg.)  
 Exp. B. 5. 10. 20. 40. g/kg. ad. h. e. v. e. d. ap. i. t. d. f. c. diff. p. i. d. (K. p. A. 2. 1/6. 2. 1/2. 3. 4. h. Exp. B. 2. 1/2. 4. 8. h.) DA. ad. NA. i. v. m. e. a. d. i. f. i. b. i. p. i. f. b. i. p. (LIN) p. i. t. (STR) b. e. m. p. h. (STM) d. i. p. h. l. e. n. (OIK) m. l. v. e. b. i. t. e. m. (LSS) m. s. i. u. m. l. t. i. f. h. m. T. l. e. m. e. d. a. d. p. l. t. i. o. n. m. d. i. K. p. A. 4. h. f. m. e. r. p. h. i. (10 mg/kg) ad. i. f. i. L. i. N. m. d. S. T. R. The. b. l. u. l. y. h. i. g. h. t. i. g. f. f. i. K. p. B. w. b. i. d. l. c. h. t. h. s. m. a. l. l. t. d. s. (5 mg/kg) t. 2. 1/2. h. i. L. i. N. (2. m. A. 0.1 t. 6.62 (P. 0.01) ad. S. T. R. (2. m. 3.02 t. 3.43 (P. 0.01) Th. ff. f. c. i. l. l. i. d. t. 4. h. (P. 0.03) i. h. d. w. i. t. h. 8. h. w. i. t. h. 10. ad. 30. g/kg. f. r. p. h. i. l. i. t. i. g. t. h. g. h. 4. h. d. d. i. p. p. i. g. i. t. h. i. 8. h. w. e. i. l. Morph. i. (10 g/kg) no. m. e. d. h. a. v. e. l. i. g. h. t. i. g. f. f. i. t. NA. d. p. l. t. i. o. n. i. D. I. X. I. L. S. d. i. f. i. d. m. e. p. l. o. n. g. e. d. i. t. i. d. l. d. y. f. 5. mg/kg. S. T. R. f. i. l. d. t. h. y. t. h. i. f. f. i. r. e. i. i. i. d. d. t. h. l. o. d. y. 5. mg/kg. f. r. p. h. i. f. f. i. t. m. e. i. m. a. l. l. y. i. l. i. m. T. l. e. d. DA. d. NA. d. p. l. t. i. (the. f. r. e. i. L. i. N. ad. S. T. R. the. l. t. i. L. S.)

Hakola, K. Attil, L.M.J. Andersson K. and M. G. K. (Res. Labs. State Al. Monopoly, Dept. Pharm. Unit, Helsinki, Finland and Dept. Med. Karolinska Inst. Stockholm, Sweden) **ON THE ROLE OF CENTRAL CATECHOLAMINE NEURONS IN ETHANOL INTOXICATION**

To study the role of central catecholamine neurons in ethanol intoxication the brain catecholamine level of rats was lowered in three different experiments. In the first study newborn rats were treated with 6-OHDA (3 100 mg/kg. 11 & interval) selective resection of brain noradrenaline. In the two experiments adult rats were used. 6-OHDA was injected bilaterally into either the ascending dopamine (8 µg/4 l) or noradrenaline (16 µg/4 l) pathway to lower the level in dopamine noradrenaline levels respectively. Endogenous noradrenaline in the neonatally 6-OHDA treated animals was selectively reduced by 100% in the cerebral cortex 35% in the midbrain and increased by 117% in the pons-medulla. In adult rats 6-OHDA caused marked depletion of the dopamine noradrenaline pathways near which it was injected. For studying motor coordination the ethanol administration (2 g/kg. i. p.) the tilting plane test was used. Neonatal depletion of brain noradrenaline caused the rats to be significantly affected by ethanol than the control when we tested the rats at four months. This was also evident if rats depleted of brain noradrenaline as adults. In contrast ethanol impaired the performance of the dopamine lesioned rats significantly less than that of the control. These findings suggest a role for the central catecholamine neurons in the intoxicating effect of ethanol.

Wibberg J.F. & M. Axelsson K.L. (Department of Pharmacology Linköping University S-581 85 Linköping Sweden) **AN EFFICIENT METHOD FOR STUDYING NEUROTRANSMITTER RELEASE IN VITRO BY THE RADIOISOTOPE TECHNIQUES**

A system has been constructed which allows the simultaneous measurement of neurotransmitter release from four independent tissue preparations by utilizing the radiochemical methods for measuring  $^3H$ -noradrenaline and  $^3H$ -acetylcholine (Wibberg 1977 Acta physiol. scand. 101: 302-317). The device consists of four perspex holders in which the preparations can be mounted. Incubation and washing of the preparations is performed by inserting the holders in scintillation vials containing the appropriate media. For this purpose 40 l is required. A stainless steel holder is a thermostated water bath. Sequential washing is performed by transferring the perspex holders to new vials. After the incubation the activity of the washing solutions can be measured directly after addition of scintillation cocktail to the vial. Oxygenation of the medium is performed through polyethylene tubing connected to each one of the perspex holders. Two platinum electrodes are also mounted to each holder allowing electrical field stimulation of the preparations. In order to allow the four preparations simultaneously using only one physiological stimulator. Cheap electronic device (physiological stimulator distributor P10) has been invented. The P10 consists of four relay relays which are appropriately timed by integrated TTL circuitry. In order to distribute the pulses from one Grass stimulator sequentially to four output channels. By this approach stimulation of all four preparations can be performed at all desired pulse durations and pulse frequencies without the need of three extra stimulators. The whole system has been tested and found to work properly both for measuring  $^3H$ -noradrenaline release from guinea pig and  $^3H$ -acetylcholine release from guinea pig tissue.



Niemi, J. and Putkonen, P. T. (Department of Physiology University of Helsinki, Finland)  
ALPHA-ADRENERGIC AGONISTS VERSUS ANTAGONISTS AND PARADOXICAL SLEEP CONTROL IN THE RAT

The  $\alpha$ -adrenoceptor stimulant lonidine (CL) appears to be a universally effective suppressor of paradoxical sleep (PS) in vertebrates from birds to man. The effect of this agent is antagonized by several  $\alpha$ -blockers including selective postsynaptic antagonists tyroxamine and phenoxybenzamine (Putkonen, P. T. et al. In: Pergamon Ed Pharmacology of the State of Art, 1979, Pergamon Press, in print). Both phentolamine (PH) and yohimbine (YO) effectively antagonized CL and PH even increased PS when given alone.

To determine the effect of  $\alpha$ -blockers on the PS-suppressing effect of CL in rats, 12-hour day-time polygraph recordings were used. CL (100 µg/kg) inhibited PS for ca 8 h and decreased 12 h values of PS from 8.2 ± 1.1 h (SD) to 3.9 ± 1.1 h ( $p < 0.01$ ,  $n = 4$  t test). Pretreatment with either PH (10 mg/kg,  $n = 3$ ) or YO (1 mg/kg,  $n = 3$ ) could significantly antagonize CL. With PH however there was a tendency to increase PS in the last 4 h of the records whereas with YO the corresponding PS was even below that of CL alone.

This is in sharp contrast to our earlier results in cats, specially with respect to PH which is an effective antagonist to the PS-suppressing action of CL even in chicks (Putkonen, P. T. and Niemi, J. This meeting). Thus in spite of seemingly universal inhibitory action of CL on PS, our results suggest notable species-differences in the pharmacological profile of central catecholamine receptors which can influence the control of PS.

E. and Johansson, G. (Institute of Physiology, University of Göteborg, Sweden)  
THE MEAL PATTERN OF THE RAT DURING RAPID EYE MOVEMENT SLEEP DEPRIVATION

Changes in the feeding schedule seem to affect the amount of following rapid eye movement sleep (REMS) in the rat (Mowrer and Bolles, 1969; Neurosci 2: 255, 1971). Correspondingly the amount of REMS within a period of 12 hours has been shown to predict food intake during the following 12 hours in the rat (Sugrue, Physiol Behav 15: 399, 1975).

In this work, study has been made of the relationship between feeding behavior and REMS deprivation in the rat.

Twenty-four hour cycles of food intake were continuously recorded in male rats on 12/12 light/dark (LD) schedule during baseline conditions and REMS deprivation.

The baseline food intake was similar to that reported by Aronson et al. (Physiol Behav 21: 785, 1978).

It took place mainly during the dark hours, with marked increase prior to the beginning of the light hours. The increase was attributable to the consumption of very large meals.

In rats which were partially REMS-deprived, the large meals were shifted toward the middle of the dark period and an increase was observed in the frequency of small meals during the light hours.

Total REMS-deprivation in adult rats replaced the well-organized meal pattern by frequent small meals distributed evenly throughout the 12/12 LD cycle. When the REMS deprivation was finished, the meal meal pattern returned.

The results suggest that the correct timing of the consumption of meals in the rat with respect to the LD cycle seems to be dependent upon undisturbed REMS. Total REMS-deprivation completely disturbs the generation mechanism for large meals.

Alihanka, J. and Kaas, J. (Department of Physiology University of Turku, Finland)  
TEMPORAL DISTRIBUTION OF BODY MOVEMENTS DURING NORMAL WHOLE NIGHT SLEEP

Temporal distribution of body movements during whole night sleep recording in ten 20-30 year old male students was studied during 20 nights EEG, EOG, EMG and subcutaneous EMG were recorded on the Statist Charge Sensitive Bed (SCSB) method was used for recording of the durations of the movements (Alihanka, J. and Kaas, J. Vastu- ja Elektroonika 11: Neurophysiol 1979, in press). The total number of movements during one night was between 80 and 200. The movements were divided into four groups according to their duration: 5s, 5-10s, 10-15s and 15s. The interval between all consecutive movements and between the movements within each group were measured separately. Interval histograms were made for each night and the whole material with less than 1 interval is 20 sec. The Kolmogorov test was used to determine the differences between the measured movement intervals and random intervals created with Poisson process. It was found that the movement intervals were not randomly distributed either if all movements of the night were considered or with any of the groups. It was also found that in the group of movements 5 intervals 1 min in the group of 5-10 intervals between 5-30 min and in the group of 15 intervals longer than 20 min were overrepresented especially 30-60 movements appeared often in series while the interval between long movements were often long. The recording of the different movement patterns provides useful method for studying cyclic physiological processes involving the motor system during sleep. Grants: The Juhon Vainio Foundation.

Eläkelä, I. and Leppävuori, A. (Department of Physiology, University of Helsinki, Finland)  
OPPOSITE INFLUENCES OF ALPHA- AND BETA-ADRENERGIC AGONISTS ON PARADOXICAL SLEEP IN THE RAT

A relatively specific  $\alpha$ -blocker phentolamine (PHE), increases paradoxical sleep (PS) in the rat (Putkonen, P. and Leppävuori, A. Acta physiol scand 1977 100: 485), whereas nonselective ( $\alpha_1, \alpha_2$ ) highly lipid soluble  $\beta$ -blocker propranolol (PRO) decreases PS (Eläkelä, I. et al. Med Biol, 1978 56: 138). Atenolol (ATE) is a  $\beta_1$ -selective, poorly lipid soluble blocker with claimed weak entry into the brain.

The combined action of PHE with PRO and ATE was studied in rat with permanent electrode for EEG, EMO and EMOG. Sixty min polygraphical records were taken after intraperitoneal injection of 10 mg/kg of PHE alone or preceded by 5 mg/kg of PRO. ATE as the two have the same  $\beta$ -blockade potency ratio.

PRO counteracted the increase of PS by PHE ( $p < 0.01$ ; t-test, d.f. 10) while ATE proved ineffective. The 16 hour values for PS (control 15.5%) were: PHE 20.7%, PRO+PHE 16.4% and ATE+PHE 22.9%.

The failure of ATE to oppose PHE is most probably due to its low access into the CNS. The binding of PRO to  $\beta$ -receptors demonstrated in rat brain membrane might be an efficient PS-disturbing factor. A recent report (Horne directly) PHE and PRO could act on noradrenaline transmission either by modulating noradrenaline (NA) release or by altering the neuronal responses to NA. A postsynaptic level in the cerebral cortex where opposite influence of  $\alpha$ - and  $\beta$ -receptors on firing rate have been described (Stern, P. et al. Br J Pharmacol 1977 59: 635).



OJ S S and Kontro, H (Depts of f Bi  
medical Sci U iv ralty f Tampere F n  
land) EFFLUX OF TAURINE FROM RAT BRAIN SYN  
APTOMES

Taurine has been demonstrated to be a neurotransmitter in the CNS. A dose-dependent increase in taurine efflux from brain synaptosomes was observed in the presence of the taurine transporter inhibitor, TBOA. This effect was blocked by the taurine transporter inhibitor, TBOA. The results suggest that taurine is a neurotransmitter in the CNS and that its release is regulated by the taurine transporter.

The present study was designed to investigate the role of taurine in the regulation of neurotransmitter release. Taurine was found to be a neurotransmitter in the CNS and its release is regulated by the taurine transporter. The results suggest that taurine is a neurotransmitter in the CNS and that its release is regulated by the taurine transporter.

Kaakkola H and Kaakkola I (Department of  
Pharmacology University of Helsinki Finland)  
CONTRALATERAL CYCLING BEHAVIOUR INDUCED BY  
INTRANIGRAL INJECTION OF TAURINE.

Taurine has been proposed to be an inhibitory neurotransmitter in the brain. The present study was designed to investigate the role of taurine in the regulation of neurotransmitter release. Taurine was found to be a neurotransmitter in the CNS and its release is regulated by the taurine transporter. The results suggest that taurine is a neurotransmitter in the CNS and that its release is regulated by the taurine transporter.

Sire A L and Kaakkola H (Department of  
Pharmacology University of Helsinki Finland)  
CENTRAL CARDIOVASCULAR AND THERMAL EFFECTS OF ARACHIDONIC ACID IN RATS  
Arachidonic acid (AA) is a precursor of prostaglandins (PGs) and thromboxane (TXA<sub>2</sub>). The present study was designed to investigate the role of AA in the regulation of neurotransmitter release. AA was found to be a neurotransmitter in the CNS and its release is regulated by the AA transporter. The results suggest that AA is a neurotransmitter in the CNS and that its release is regulated by the AA transporter.

K. I. Kaakkola S and Al. K. I. M. M. (Dept. of  
Pharmacology University of Helsinki Finland)  
EFFECTS OF SOME 6-CARBOLINES ON PHENETHYLAMINE AND APOMORPHINE STEREOTYPES IN RAT  
Acridine derivatives (ACR) are a class of compounds that have been found to have effects on neurotransmitter release. The present study was designed to investigate the role of ACR in the regulation of neurotransmitter release. ACR was found to be a neurotransmitter in the CNS and its release is regulated by the ACR transporter. The results suggest that ACR is a neurotransmitter in the CNS and that its release is regulated by the ACR transporter.





K mg L Ml l A t, Lami t pvt R  
L pph A d Niamie L (F rm s Group  
Ltd Res rch C t L ske-Medipol s d Be-  
partme t of Pharma legy Univ rsity of Turk  
F l l d): DISTRIBUTION OF METOPROLOL BETWEEN  
PLASMA AND SALIVA

Metoprolol (M) bet blackf g ge t s  
determi ad i pl a d salt of 10 h lthy  
ol tears fo 0 22 h a well as in pl sma  
s liva d s lly ary gla d of 3 at 3 h after  
a i gl admi istrati p e Th kema dose  
s 100 mg d the ant i dos 80 mg/kg

The co est t l of M were det rm ad by  
GLC usi g t s electic det to  
Th salt ry l l f M ere egul ly and  
sig ific tly highe th th plas m e als  
F th pe k le l in l t s were 846  
156 d 121 32 ng/ml (we n t ) Th me  
ratio salt a/pl sma of all a epl s w s l  
Th l l t i c k l f lfe us si l l in both  
f l id (we a 2 6 and 2 0 h, resp ctively)  
Th l als of M in at pl ma ere 93 646  
(we 384) g l Highe a trat ere  
found i aliva d l saliva y gla d s va gi g  
260 853 d 250 837 ng/l r pe tly Th se  
f l id g M th t l) M l cewl t g te  
l ly i t th l f r glands d 2) dist i  
bet l f M betw plas d ali o t k  
t pla by imple pe l diff i b t  
thro gh bi ding t th lly y gl d

Osage G, Jacobsen P and Kansteen V  
(In titte of Pharmacology Univ ity f Oslo  
and Medi l Department VIII Ullevaal Hospital  
Oslo Norway) ANTICOAGULATION AND PLASMA  
PROTEIN BINDING OF PROPRANOLOL

Propranolol is extensively bound in plasma  
acid glycoprotein (AGP) i the mal bi-  
ding protein but propranolol i also distri-  
buted to lipoproteins [1] These two protein  
f ctions ar respon ibl f 60-75% and 5-25%  
of total binding respectively Binding f  
propranolol wa tudied in plasma from 15 pati-  
ts receiv ing anticoagulation the apy (war-  
farin or heparin) after myocardial inf rctioe  
Plasma binding wa d termined t propranolol  
concentration of 125 ng/ml by equilibrium dia-  
lysi against Krebs Ringe buffer pH 7.40  
32°C Free f ctty acids (FFA) were determined  
by col isetric method [2] The unbound  
f ctions in plasma were 0.065 0.013 and  
0.078 0.015 ( 2 5%) and the FFA level were  
0.6 0.4 and 1.9 0.7 meq/l ( 2 5%) after war-  
farin and heparin administration respectively  
Increase in FFA i rpl ined by heparin acti-  
vation f lipoprotein lipase (LPL) Positive  
linear correlation was found between unbound  
fr ction and concentration f FFA ( 2 5%) In  
vitro addition f heparin or FFA [3] did not  
affect the binding Concentrations f AGP  
were not significantly diff rent i the two  
groups ( 5%) The present results indicate  
that the activation f LPL decreases lipopro-  
tein binding of propranolol

Ref rence 1 Sager G Nilsson O G and  
Jacobsen P Biochem Pharmacol (in press)  
2 Leuverye R R Analgt Biochem 32 331  
(1969) 3 Spector A A and Meak J C Ana-  
lyt Biochem 32 287 (1969)

And wa on P, Bjerregaard P, Kordeus H J  
Linderholm Ped reen O Mi kelsg E, Peders-  
sen K B M Week 3 (University f Aarhus  
Denma k) BIOCHEMICAL CHANGES DURING PROPRANO-  
LOL TREATMENT

Thirty patients ( women and 16 men) with WHO hyper-  
tense on were readonly alloted to one f three groups  
treated during 8 weeks with propranolol in preceeding  
run-in period the dose was gradually increased to 40 mg  
4 times dail (group 1) 80 mg 4 times dail (group 3)  
or 160 mg times dail (group 3) The ave age plasma  
concentrations of propranolol (af or the 4 week adjust-  
ing period after weeks and after 12 weeks) were 66  
75 78 76 and 54 54 64 ng/ml ( 2 ) in group 1  
71 137 183 15 and 162 123 in group 2 and 296  
137 390 11 and 140 37 ng/ml in group 3 The pro-  
tein concentration in serum decreased in 9 f the 10 pa-  
tients in group 1 No consistent changes were observed  
in the two other groups On the average the concentr ti-  
on f creatinine in serum increased with 0% The crea-  
tinine clearance decreased in group but d no change  
inpl ctly in groups and The concentration  
f and wa in serum and its excretion in the urine di-  
not show any consistent changes whereas the potassium  
concentr tion serum ose the two groups given the  
lower doses not remained unchanged in the high dose  
group (all women 75 00 all men 08 30 30 ml/l)  
Serum cholesterol showed no change tr lywise  
e-vals increased in groups 2 and 3 Serum uric acid con-  
centrations showed an average incr ase de al three  
groups from 5.0 to 5.4 mg/100 l The percentual increas-  
e was highest in group 1 and low group 3 y-con-  
centration in plasma l l in all three groups with an  
average f 20%

Actioe/poosms Supported by the Danish Heart Associa-  
tioe.

S md R R So g al sed l ssa 3, (Be-  
partme t f barn ecology ad Ph reace ti l  
Chemist ry l t s f Ph reacy alive i y f  
Osl Herv y) ILIARY AND URINARY EXCRET ON OF

SO CO LY DERIVATIVES IN THE RA  
llowing p tedi xyphe l d i  
ti ( ad l as ph rae pl exicel  
1979 258) ylk oodyl (I f d l  
phenal) and its di t ith wa i 14 ( l  
i oodyl) and lphari 14 (222-pl sulp)  
ve give by tesa k tube f ed  
d 2 l grol pe 100 g l cell ed  
i th go ied O h 6 12 h and 12 18 h f llw  
ing dail ti ad y 8 to al l  
f p jag ted) by LC thed ith  
l pe i l and d Mo f i as  
( f d t ) ed i s pe sen-  
po ad ad peried wa 41 2 4 3 8 8 14 7  
and 25 l 2 5 74 0 4 7 51 9 2 7 9 ad  
30 8 2 3 2 5 0 2 0 9 28 0 2 5 4 ad 19 6  
3 l aly iso amocate wa p f  
Sampl k by biad p ar ad y  
ed above thermo hved th l lary  
er amo f l i ignifie compared  
amo ox ed i bil Mo I ve found  
ine 0-6 h af ad i l ion f i  
7 meol pe 100 g i fed sed d l  
he mo s ly he lthe ad i The  
re lts d mo tr tha l ad i ff lve  
ly ab bed d i g th l wall i tical tras  
i ad ed in the bil Th be rtyies  
f however tak l ce mo d taily i  
l ompl to ad i p eably doe t saymati  
hyd ly i 2 th p edrug ti l i e-  
dan th the sumpti f th l  
(Ar h Ph rae l 1972 24 6)

228

Lecms, P.V., Pelkonen, R.O. and Potjanski, E.A. (Clinical Research Unit, Department of Medicine and Department of Pharmacology, University of Oulu, Finland) PLASMA HIGH-DENSITY LIPOPROTEIN CHOLESTEROL AND HEPATIC DRUG METABOLIZING ENZYME ACTIVITY IN MAN

Recent studies have linked high-density lipoprotein (HDL) cholesterol with the development oftherosclerotic diseases. Plasma HDL cholesterol level is in inverse relationship to the incidence of coronary heart disease. Some compounds such as alcohol and phenytoin increase the plasma HDL cholesterol level. The effect might be related to the stimulatory effects of these compounds on the activity of hepatic microsomal system. To test the hypothesis we compared plasma HDL cholesterol level with hepatic cytochrome P-450 content and with the rate of antipyrine elimination from plasma in patients with diagnostic liver biopsy.

The results showed positive correlation between plasma HDL cholesterol level and cytochrome P-450 content in the liver and also between HDL cholesterol and the antipyrine elimination rate. Plasma HDL cholesterol and hepatic cytochrome P-450 concentrations were related to liver histology.

The results suggest that there is relationship between the hepatic production and the plasma level of HDL cholesterol. The findings also suggest that hepatic cytochrome P-450 reflects liver capacity for both HDL cholesterol production and drug hydroxylation.

Grant: Finnish National Research Council for Medical Science (Academy of Finland)

228

Salonen, J.S. (Department of Pharmacology, Institute of Biomedicine, University of Turku, Finland) PLASMA LEVELS OF PROCAINAMIDE AND N-ACETYLPROCAINAMIDE DURING MAINTENANCE THERAPY

The steady-state levels of procainamide (PA) and its active metabolite N-acetylprocainamide (NAPA) in plasma were studied during maintenance therapy with sustained release PA. Both concentrations were simultaneously determined using gas-chromatography with nitrogen sensitive detection. Samples from 40 hospitalized patients receiving an average daily dose of 2500 mg PA (range 800-3600 mg) were analyzed. The patients ages varied between 42 and 81 years (mean 61 years) and majority of them was suffering from myocardial infarction.

The steady-state PA level at the time of dosage ranged from 0.5 to 10.5 mg/L and the NAPA level ranged from 1.4 to 16.8 mg/L. Below the proposed therapeutic range 4.18 mg/L (Koch-Weser, Clin Pharmacokin 1:189 1977) were 73 and 54, respectively. Concentrations less than 0.5 mg/L were found during the absorption phase only. The NAPA level ranged from 0.5 to 10.6 and from 0.6 to 18.7 mg/L respectively. The acetylation ratio C(NAPA)/C(PA) ranged from 0.4 to 3.7 and was less than 2 in 77% of samples. The time of PA dosage.

Many of the patients were receiving reduced PA dosage because of renal dysfunction. Heart insufficiency. However, there seems to be a light tendency towards subtherapeutic plasma concentrations of PA, which would indicate the need for more regular plasma level determination. Conclusions about the contribution of NAPA to the therapeutic efficacy adverse reactions in these patients all have to wait for further investigation.

227

Sepponen R., Järnäs, R., Viikari J. O. Anttila, L. and Viikari J. (Department of Pharmacology and Internal Medicine, University of Turku and Research Center Lääke-Medipoli Turku) STEADY STATE PLASMA LEVELS OF CHLORPROPAMIDE AND ANTIDIABETIC RESPONSE IN DIABETIC PATIENTS

Chlorpropamide (CP) is used in the therapy of diabetes insipidus and it is also known to induce an inappropriate ADH-secretion syndrome in some patients receiving it as an antidiabetic agent. The purpose of this study was to investigate whether there is correlation between steady state levels of CP and arginine vasopressin (AVP) in patients (n=23) having diabetes mellitus. CP was given 125-750 mg/day. CP was measured spectrophotometrically and in some cases also gaschromatographically (GLC). As previously known the interindividual variation

of the CP plasma concentration after equal doses was large. The plasma levels ranged from 31 to 211 µg/ml (mean 118±54 µg/ml). The specific GLC procedure gave in low concentrations similar results but in high concentrations the GLC tended to give lower values. The value of AVP ranged from 2.8 to 21.3 pg/ml (mean 10.6±5.3 pg/ml). The mean AVP value of these patients did not differ significantly from the mean fasting value of the controls (9.7±4.5 pg/ml, n=23). No correlation between plasma concentrations of CP and AVP was found (r=0.22). Others have shown that CP has an antidiabetic action, i.e. in some normal persons and diabetic patients CP has been suggested to act by increasing the synthesis and release of ADH. In this material of diabetic patients no significant change in the plasma ADH level was found during the chronic administration of CP.

229

Sepponen M.I., Myllylä V.V. and Leppä P.V. (Departments of Neurology and Radiology, University of Oulu, Oulu, Finland) INTERACTION OF SODIUM VALPROATE AND CARBAMAZEPINE AS REFLECTED IN SODIUM DRUG LEVELS

Sodium valproate (VPA) is now widely accepted as an effective drug in petit mal epilepsy and also in grand mal epilepsy especially in combination with the anti-convulsants. The major pathway of metabolic transformation of VPA is conjugation with glucuronic acid. The major pathway of metabolism transformation of carbamazepine (CB) after treatment with steady state level of drugs in the body. To prevent drug interaction it is important to evaluate the antiepileptic therapy with the combination of VPA and carbamazepine (CB) as reflected in serum drug levels.

Serum VPA level was determined by enzyme immunoassay and that of CB by gas-chromatographic method. Therapy with VPA alone resulted in high VPA level than the measured in patients who were treated with VPA in combination with CB. Serum VPA level in patients treated with the combination was in most cases at subtherapeutic level. Serum CB level in patients treated with the combination of VPA and CB or with CB alone did not differ from each other.

The results suggest that CB enhances the clearance of VPA from the body. In this connection low VPA level may reflect the CB-induced stimulation of hepatic metabolism of VPA.

Grant: Finnish National Research Council for Medical Science (Academy of Finland)



242

Klyne, R. Geisler A. Hansen R.W. Stahl Skov, P.  
and Rør, S. (Department of Pharmacology, University of  
Copenhagen, Copenhagen, Denmark): HISTAMINE H<sub>2</sub> RECEPTOR-  
RELATED CYCLIC ADP FORMATION IN HUMAN PLATELETS

Histamine-sensitive adenylyl cyclase has been described in various tissues. Thus, histamine increases cyclic AMP formation in gastric mucosa, uterus, heart, fat cells, and leukocytes by interaction with its receptor-linked adenylyl cyclase. This effect is blocked by  $H_1$  antagonists; e.g., cimetidine. In brain, it appears that both  $H_1$  and  $H_2$  receptors are associated with the formation of cyclic AMP. It is known that the effect of histamine on cyclic AMP formation in human platelets has not yet been studied. We have observed that histamine increases the cyclic AMP level in human platelets in dose-related manner. Maximal stimulation was obtained with  $10 \mu M$  histamine usually giving two-fold increases above basal values. Half maximal activation was achieved with about  $1 \mu M$  of histamine. An increase in the cyclic AMP accumulation was obtained with concentrations as low as  $0.01 \mu M$  of histamine. This histamine-induced cyclic AMP formation could be blocked by the  $H_2$  receptor antagonist cimetidine whereas the  $H_1$  receptor blocking agents phenhydramine and diphenhydramine were unable to antagonize the histamine response. Methysergide had any effect on the unstimulated cyclic AMP level. The histamine-induced cyclic AMP response was unaffected by propranolol as well as by the 1 $\alpha$ -methyl-phenylethanolamine. These results suggest that histamine can elicit the formation of cyclic AMP in platelets by stimulation of  $H_2$  receptors. Since the platelet preparation was contaminated with only few leukocytes (4.0%), which are known to possess  $H_2$  receptors, the histamine-induced response will be controlled with more purified platelet preparations.

243

Tuomisto L. and Tuomisto J. (Dept. of Pharmacology, Univ. of Helsinki and Dept. of Pharmacology and Toxicology, Univ. of Kuopio, Finland) MONOMINE UPTAKE AND RELEASE IN RAT BRAIN SYNAPTOSOMES: EFFECTS OF HISTAMINE AND HISTAMINE ANTAGONISTS

H<sub>1</sub> histamine receptors adsorb to the adrenal medulla and both H<sub>1</sub> histamine and its antagonists have been used to release monoamines from CNS neurons. Hence it was studied whether or not this effect was direct tyramine-like release (i.e. the level of the nerve ending). Rat brain synaptosomes (P<sub>2</sub> fraction) were incubated in Krebs-Henseleit buffer with 0.1 μM tritiated monoamines. Hypothalamic synaptosomes with 5-HT, cortical synaptosomes with dopamine and cortical synaptosomes with noradrenaline. For studying uptake inhibition various concentrations of histamine or cimetidine were added. Methods of assay were: verapamil, acetic acid or imipramine (reference) were included in the incubation media. For studying release the synaptosomes were preincubated for 5 min with the substrates and washed by centrifugation and resuspended and used for second incubation with the compounds studied. In two uptake experiments only imipramine was found to be a potent inhibitor (IC<sub>50</sub> was 1.5 μM (DA) to 30 μM (5-HT)). Barbiturate-inhibited monoamine uptake at 0.15 μM and others were even less potent. Verapamil, acetic acid was completely inert. Release experiments with histamine induced some release both at 1 and 5 μM incubation but these concentrations were needed in 12 orders higher than those of tyramine. Both histamine and antagonists were inert even at high concentrations (1 mM). Hence the results do not give support to the possibility that histamine or the H<sub>1</sub>-antagonist to burst monoamine or cimetidine influence monoamine uptake or release. It remained concentrations by tyramine-like release in the nerve ending.

244

Øyul & G. Hjort M and Røssum E (Institute  
of Medical Biology and Instit of Mathemat-  
ical and Physical Sciences University of Tromsø  
Norway) THE THREE DIMENSIONAL MOLECULAR STRUC-  
TURE OF ACTIVE AND INACTIVE PHENOTHIAZINE METAB-  
OLITES

It has been demonstrated that psychiatric patients on or 1 treatment with levomepromazine have higher plasma level of the sulphoxide metabolite than of the parent drug (Dahl & G. Cilia Pharmacol Ther 1976 12 435). Levomepromazine sulphoxide had cardio-depressive effects isolated rat atrial muscle which we compared to the effects of the parent drug while the sulphoxide of chl promazine was virtually inactive in this as in other systems (Dahl & G. and Refsum & Europ J Pharmacol 1976 27 241).

241) This study was carried out in order to reveal any characteristic features of the molecule structure which could distinguish active from inactive phenothiazine metabolites. The three-dimensional molecular structures of levomepromazine sulphoxide and 8-chloropromazine sulphoxide were examined by single-crystal X-ray diffraction.

The molecule structure of levomepromazine  
aliphoxide vs imide the previously pub-  
lished structure of chl ipromine (McCowell, J  
J, Acta Cry 1969 21: 2175) is chl iprom-  
ine sulphoxide however the ide that has an  
entirely different conformation in the solid  
state This might expl in the low pharmaco-  
logical activity of chl ipromine aliphoxide  
compared with levomepromazine aliphoxide and  
possibly other drugs

245

HY-BLOCKS AFTER AMITRIPTYLINE AND METECOPOL  
AMINE IN UNANESTHETIZED BEAGLE DOGS

Amitt ipytline (ANI) h  
2 d d g AV bl h  
[1] W h fe d h t f  
i f t f ANI od d d gree AV blocks  
i 6 out f ? (ANI) Th i f th  
t dy t inv tig t h the p t  
rel t d t th ffect f ANI ths ble k  
ystem M th c polamin (H) us h li gic

ARI g gl on hy i faest f 1D i t  
O S g/kg/ f EGG nd ho t were ti t  
nely ita d At i t two week l t th  
n me dogs wa Bl M O DIS mg/kg i f flowed  
18 28 l t t by AM i f aion Th d g were  
t d d  
Bu i f AMI i f t l De fed f 2nd d g av  
bl ks l t d f b-r d fca 3-6 d S i t  
t l t d f 1-6 d f M us d p lod f 2 d  
d g AV bl ks 3-4 f Et t i j t t  
t l t f 1-3 i The bl ks c d hortly  
bef al itanous ly with d l ping  
t by rdl i f ina f AMI fter M did not  
2nd degr AV ble k  
Th on t indi t  
g ee AV bl k by i t that AMI may av 2nd  
ph l h line g t t l s ith th perf  
l El E Et al E J Ph rm i 78  
188 1874

Komul en H and Tsonit t, J (Dept f  
Pharma l nd T l l, Unl { Kuopl  
FI land) EFFECT OF COPPER ON THE UPTAKE AND  
RELEASE OF DA MA AND 5-HT IN RAT SYNAPTOSOMES  
IN VITRO

Many t l neurot x in CNS but t  
ch i s of to icity known Beh i l  
nd went i d turb acce ften acc thout  
y g pathological damage i th brai  
Po ble mecha low includ modulation f euro  
tra swit pt k and/ l f om t rag  
it s i rv end g We have studied th  
ff t f one (a CuCl2) i vit on th  
l tit l upt k and relea f tit ited DA MA  
nd 5-HT) t c i c i o c l i nd Hypo  
thalas vnapot ome specti ly Incub tion  
we i d out ith o μM sub trat i  
K b H l t bi boot buff pH 7.4 i  
37 C and t rained ith wash and filter tion  
ICgo t 2 μM cubat on ver o μM (MA)  
i d o μ (DA) and o μM (5 HT) Th inhibit f  
DA and 5-HT upt k we no competit time and  
ncent t on dependent nd cress th  
p cub t i (i l) f ynapot ome ith Cu<sup>++</sup>  
M pt l wa i bibic d by l but inc ed by  
high ( 10 μM) on eat ti P i cub ti  
d ed the h bit on f MA upt k With  
50 μM th high fff ty upt k f MA wa  
t l l inc ed (t 2 l) but l t  
dec ed Inc ed upt k we sea l t  
OC th t may be dgt c owe pa f oc  
po hap bel t C t i duc d lse f DA  
MA and 5 HT i q ifl cly th po t eou  
i f om l d d vnapot ome The  
echan as f t f oppo enai t b  
tnd ed but t l t gu e that th co extra-  
eed d q t low and th ane  
po bl ed espe lly i M l di esse  
G t 4c dew f f l and

273

Saarni, H.U. and Pelkonen R.O. (Department of Pharmacology, University of Oulu, Finland) THE METABOLISM AND DNA BINDING OF BENZO(a)PYRENE CATALYSED BY HUMAN PLACENTAL MICROSOMES

Aryl hydroc rben hydroxyl as (AHH) activity i  
equilish in ploc etes from noncoking methers bet  
may be leavert w 100-f id i placerte from ci  
garrett smok re Becuase AHH i thought t be reson  
nabl f the activation f polycycl aromatic  
hydroc rben t metegol and wringnoll f re ve  
become int tested in see wring the ab ility f buses  
placert to catalyze the in fire metebellon and DNA  
binding f benzol pyrene (BP) Placental i rosmare  
14-BP purified Salaman perra DNA and nec very o  
fact re were incubated and thereaft the DNA was  
precipitated pu ified, hydrolyzed and nucleo ide-  
metebell compl chromatographed by Sephad Lit-  
20 Incubet were tracted by thyl ac tot and  
net bollite ptt re measured by HPLC Phenol sul  
none and one dihydrodi i (see ibly 7-8 di i) meteb-  
helt were detected Both 9,10 di i and 4,5 di i  
i were com pl wocly lacking BP metebell were bound  
t DNA gling fte t two ml peak h i h taster  
vely were identified acetoaloxide sepl f 7-8-  
9,10-epoxide and 7,8-oxide and / quinone As  
compared t binding ptt re aut lyzed by rat live i  
rosmare nuclea ide-metebell peak att ibuted t  
4,5-oxide and 9-phenol-4,5-oxide were lacking Re-  
it deman trst that both the metebelt ptt re  
and nuclea de-metebell compl produced by pla-  
ental microsomes f liver diff greatly from those  
produced by rat live i rosmare The test i binding  
f BP metebelt t DNA serrulated guffi att ly  
ith both fluoromet ally neo red AHH att ility  
and the amount f the di i metebelt f reed These to-  
di f rthe enfirm that t i not po bl to  
predi t the pssibi ptt ree f DNA binding from  
AHH mea rument even from BP metebelt to ptt re



Hyvärinen, J. 79, 182, 183  
 Hyvärinen, L. 183  
 Häily, M. 101  
 Häkkinen, R. 18, 112, 139, 146  
 Hänninen, O. 277, 279, 280  
 Härkönen, R. 64  
 Höglund, A. U. 138  
 Hökfelt, T. 143, 147, 170, 198

# I

Ihalainen, E. 258  
 Iisalo, E. 217  
 Iobekk, A. 44-46  
 Ingemann-Hansen, T. 84, 81  
 Ikonen, R. 176  
 Iversen, J.-G. 3

# J

Jacobsen, S. 223  
 Jahnén, H. 164  
 Jakobson, A. 235  
 Jansen, J. A. 234  
 Jansen, P. K. 121  
 Jansen-Holm, J. 162  
 Jodal, M. 110  
 Johansson, G. 136, 192  
 Johansson, L. 147  
 Johansson, P. 210  
 Johansson, S. A. 218  
 Jonsson, G. 198  
     J-O. 210  
     F. 180  
 Jørgensen, K. E. 128  
 Järhult, J. 21  
 Järvenheimo, P. 221  
 Järvelä, M. 136  
 Järvelä, T. 30

# K

Kaakkola, S. 207, 253  
 Kangas, L. 215, 222  
 Kanto, J. 213  
 Kao, F. F. 88  
 Karl, I. 208  
 Karlberg, L. 123  
 Karonen, S. 126  
 Karppinen, H. 208, 246, 247  
 Kauppinen-Waara, K. 102  
 Kaurela, M. 76  
 Kattunen, R. 63  
 Kien, R. 85  
 Kiesling, K.-H. 33  
 Kiernä, K. 176  
 Kik, F. 1, 44-46  
 Kinnula, V. 113, 156  
 Klinge, E. 76  
 Klyner, R. 241, 242  
 Knap, M. 144  
 Kohvasalo, F. 144  
 Komulainen, H. 271

Kontro, P. 206  
 Kopp, V. 131  
 Korneus, H. J. 224  
 Korpi, E. R. 173  
 Koskela, M. 133  
 Koskimies, A. 196  
 Kousu, N. 213, 214  
 Krog, J. 164  
 Kuitila, E. O. 49  
 Kulkka, J. T. 49, 62, 72  
 Kyötilä, J. 270  
 Käskö, O. 123  
 Kärd, N. 26, 281  
 Kärkkäinen, L. 207

# L

Lagerström, K. Y. H. 103  
 Lahti, H. 188, 186  
 Laitinen, A. 168  
 Laitinen, M. 277, 278  
 Lammintausta, K. 71  
 Lammintausta, R. 71, 163, 213-  
     215, 222, 227  
 Landgren, S. 165, 187  
 Landmark, K. 220  
 Larsen, J. A. 128  
 Larsen, T. 86  
 Larsson, C. 205  
 Larsson, M. 122  
 Lautala, P. 161  
 Leander, S. 148  
 Lederballe Pedersen, O. 224, 239  
 Lehtomäki, M. 79  
 Lehtonen, M. 39  
 Lehtovirta, P. 76  
 Leino, R. 227  
 Leinonen, A. 104  
 Leinonen, L. M. 182  
 Leinonen, M. 133  
 Leinen, J. 44  
 Leppiluoto, J. 144, 146  
 Leppänen, M. 222  
 Leppävuori, A. 183  
 Lewander, T. 187  
 Libelius, R. 84  
 Lie, B. N. 119  
 Lindahl, B. 107  
 Lindbom, L. O. 238, 246  
 Linden, I. 8, 232  
 Linnekoski, I. 183  
 Lisander, B. 17  
 Lonin, T. 18, 139  
 Lundberg, J. M. 19, 21, 143, 147  
 Lundborg, P. 99  
 Lundgren, B. 114, 237  
 Lundgren, O. 110  
 Lundholm, E. C. 289  
 Lundvall, J. 76  
 Luoma, P. V. 219, 226, 229  
 Lystad, E. 3  
 Lähde, S. 47  
 Lähmies, E. 72

# M

MacDonald, E. 258  
 Macdonald, P. I. 279  
 Malström, J. 238  
 Malmsten, J. 106  
 Manninen, H. 151  
 Marttila, M. J. 100  
 Masoli, J. 129, 130  
 Maasilta, J. 157  
 Menard, R. H. 28  
 Merilä, M. K. 150  
 Meyer-Rochow, V. B. 179  
 Meyerson, B. J. 4, 136  
 Mikkelsen, E. 224, 239  
 Miller, M. M. 46, 48  
 Milton, A. 124  
 Minneman, K. P. 61  
 Mäse, O. D. 48, 50  
 Modigh, K. 137  
 Moen, P. 86  
 Mollanen, M.-L. 26, 275  
 Moldau, P. 24  
 Molinoff, P. 61  
 Muittari, A. 100  
 Myrskylä, V. V. 219, 229  
 Mäntä, J. 36  
 Mäkelä, J. 190  
 Mäkeläinen, M. 88

# N

Neibert, D. W. 28  
 Nedergaard, O. A. 68  
 Newgard, C. B. 140  
 Nielsen, C. K. 68  
 Nielsen, H. 90  
 Nielsen, M. 11  
 Nielsen, S.-L. 73, 74  
 Nielsen-Kudsk, F. 230  
 Nieminen, A.-L. 222  
 Nieminen, L. 215, 222  
 Nieminen, M. 133, 278  
 Nieminen, M.-L. 248, 252  
 Nieratsch, W. 25  
 Nilsson, J.-E. 66  
 Nilsson, G. 112, 141-143  
 Nil, A. 167  
 Nordberg, A. 163, 203, 205  
 North, E. 181  
 Norton, B. J. 123  
 Norling, A. 280  
 Norm, S. 241, 242  
 Nuutinen, M. 61  
 Nygaard, E. 80

# O

Ostlund, B. 124, 125  
 Oja, S. 8, 172, 173, 206  
 Oksala, H. 281  
 Oksa, L. 106  
 Olger, L. 142  
 Olson, N. 73

# The figures refer to the abstract numbers

Olsen, U. B. 69  
Olsen, K. A. 105, 187  
Osterberg, J. 67  
Ossi, A.-L. 247  
Ossi, K. 24  
Ossi, S. 24  
Ossi, J.-B. 62

P  
Pakkari, I. 247  
Pakkari, P. 347  
Pakonen, M. K. 10, 247, 248, 252  
Pakola, T. O. A. 66  
Pakarinen, J. 257  
Pakinen, M. 217  
Pakinen, E. B. 224  
Pakinen, A. 71, 258  
Pakinen, O. 25, 221, 225, 258, 271, 274, 281  
Pakinen, E. H. 200  
Pakinen, S. 83  
Pakinen, B.-E. 127  
Pakinen, E. 127, 239  
Pakinen, K. 88  
Pala, H. 42, 43  
Pala, R. S. 118  
Pala, J. P. 258  
Pajala, T. 281  
Pajunen, M. A. 72  
Pajunen, E. S. 48, 220  
Pajunen, G. 216  
Pala, P. 255  
Pakonen, P. T. S. 189, 190  
Pakunen, J. 115  
Pajunen, J. 118  
Pajunen, K. 257, 258  
Pajunen, A. 156, 180  
Pajunen, D. 183

R  
Rajaniemi, H. J. 144, 180, 181  
Rajaniemi, M. 47  
Rajala, S. 239  
Rajala, H. 28  
Rajala, A. 258  
Rajala, R. 135  
Rajala, H. 45, 50, 220, 252  
Rajala, K. L. 212  
Rajala, O. B. 101  
Rajala, M. I. 229  
Rajala, T. 32  
Rajala, T. 103  
Rajala, H. 185, 187  
Rajala, S. 148  
Rajala, S. 79  
Rajala, J. 184  
Rajala, L. B. 2  
Rajala, N. 61  
Rajala, H. 245  
Rajala, B. 108  
Rajala, A. 87  
Rajala, A. 148

S  
Saarela, S. 158  
Saarela, V. 97  
Saarela, S. 76  
Saarela, H. 103  
Saarela, H. U. 273  
Saarela, T. 171  
Sager, G. 223  
Sager, S. 143  
Sager, S. 64  
Salmela, P. 270  
Salonen, J. B. 228  
Salonen, B. 83, 84, 90  
Salonen, B. 225  
Salonen, O. 3, 31, 134  
Salonen, M. 37  
Salonen, R. S. 239  
Salonen, J. 8  
Schulzberg, M. 147  
Schwartz, R. 170  
Secher, N. H. 81-83  
Selin, L. C. 94  
Selkälä, H. 145  
Sella, A. 136  
Sella, M. 71  
Sella-Harjula, G. 77, 135  
Sheth, M. I. 129, 130  
Sheth, N. 25  
Sheth, R. 147  
Sippola, T. 276  
Sheth, A.-L. 208  
Sheth, G. 80  
Sheth, L. 141  
Sheth, C. 85, 86, 88  
Sheth, M. 118, 120, 122  
Sheth, S. E. 108  
Sheth, T. 62  
Sheth, P. S. 241  
Sheth, O. 60  
Sheth, E. 248, 252  
Sheth, A. 188  
Sheth, K. 225  
Sheth, E. A. 221, 228, 258  
Sheth, A. R. A. 79  
Sheth, B. 238  
Sheth, C. 194  
Sheth, P. 242  
Sheth, F. 272  
Sheth, O. 62  
Sheth, K. 121  
Sheth, H. 205  
Sheth, L. 178  
Sheth, O. 45, 48  
Sheth, M. 57  
Sheth, B. 225  
Sheth, T. 42, 43  
Sheth-Kuronen, B. 255  
Sheth, G. 106  
Sheth, F. 18, 112, 135, 148  
Sheth, A. 163  
Sheth, H. 221  
Sheth, I. 111  
Sheth, E. 78

Svensson, L. 131  
Svensson, T. H. 16, 70, 198  
Silva, V. 85

T  
Tahala, T. 68-81  
Tahala, G. 85  
Tahala, L. 147  
Tahala, E. O. 72  
Tahala, A.-E. 181  
Tahala, S. 94  
Tahala, O. 6, 125  
Tahala, P. 15, 70  
Tahala, N. A. 149  
Tahala, J. 45  
Tahala, T. M. 283  
Tahala, J. 83, 85, 133  
Tahala, A. R. 78  
Tahala, H. 25  
Tahala-Pakinen, C. 149  
Tahala, P. 251  
Tahala, R. 251  
Tahala, E. 251, 253  
Tahala, P. 251  
Tahala, M. 145  
Tahala, J. 243, 251, 253, 271  
Tahala, L. 243  
Tahala, D. 110  
Tahala, O. 67  
Tahala, S. 94  
Tahala, H. 258

U  
Ullberg, N. 161  
Ullberg, H. R. 12, 119, 120  
Ullberg, P. 25  
Ullberg, H. 9  
Ullberg, R. 194  
Ullberg-Wallersten, K. 21

V  
Valio, H. 27, 254  
Valio, K. 118  
Valio, A. B. 181  
Valio, P. 188  
Valio, R. 72  
Valio, M. H. 132  
Valio, H. 257, 258  
Valio, V. M. K. 223  
Valio, J. 227  
Valio, O. 153, 227  
Valio, H. 50, 85  
Valio, V. 184  
Valio, R. 217, 221  
Valio, M. 84  
Valio, K. 274, 275  
Valio, S. 64





ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 474

ON INTEGRATIVE MOTOR FUNCTIONS  
IN THE CAT'S SPINAL CORD

by

HANS FORSSBERG

STOCKHOLM 1979



ACTA PHYSIOLOGICA SCANDINAVICA

*Supplementum 474*

ON INTEGRATIVE MOTOR FUNCTIONS  
IN THE CAT'S SPINAL CORD

by

HANS FORSSBERG

STOCKHOLM 1979



ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 474

FROM DEPARTMENT OF PHYSIOLOGY III  
KAROLINSKA INSTITUTET STOCKHOLM SWEDEN

# ON INTEGRATIVE MOTOR FUNCTIONS IN THE CAT'S SPINAL CORD

by  
HANS FORSSBERG

STOCKHOLM 1979



## CONTENTS

GENERAL INTRODUCTION	5
METHODOLOGICAL CONSIDERATIONS	7
RESULTS AND DISCUSSIONS	10
A Spinal Locomotion	10
a) Generation of cyclic locomotor activity in a single hindlimb	11
1 Movements	11
2 Muscular activity	12
3 Force	16
4 Speed adjustments	17
5 Central generation	17
6 Peripheral control of the locomotor generator	18
b) Interlimb coordination	19
c) Concluding remarks	21
B Placing Stepping and Hopping reactions	23
a) The reactions	23
b) Basic organization in the spinal cord	24
c) Supraspinal control	25
d) Development of the control	28
e) Modifiability of placing reaction during different conditions	28
C Stumbling Corrective Reaction	30
a) The reaction	30
b) Receptors and receptive fields	31
c) Reflex pathways	31
d) Differences of responses in intact and chronic spinal cats	34
e) Phasic gating	34
GENERAL DISCUSSION	42
SUMMARY AND CONCLUSIONS	43
ACKNOWLEDGEMENTS	44
REFERENCES	45



This thesis mainly constitutes a summary of the following articles

- I FORSSBERG H GRILLNER S & HALBERTSMA J 1979 The locomotion of the low spinal cat 1 Coordination within a hindlimb Acta Physiol Scand In press
- II FORSSBERG H, GRILLNER S HALBERTSMA J & ROSSIGNOL S 1979 The locomotion of the low spinal cat 2 Interlimb coordination Acta Physiol Scand In press
- III FORSSBERG H GRILLNER S & SJOSTROM A 1974 Tactile placing reactions in chronic spinal kittens Acta Physiol Scand 92 114 120
- IV FORSSBERG H GRILLNER S & ROSSIGNOL S 1977 Phasic gain control of reflexes from the dorsum of the paw during spinal locomotion Brain Res 132 121 139
- V FORSSBERG H 1979 Stumbling corrective reaction A phase dependent compensatory reaction during locomotion J Neurophysiol 42 936 953
- VI ANDERSSON B FORSSBERG H GRILLNER S & LINDQUIST M 1978 Phasic gain control of the transmission in cutaneous reflex pathways to motoneurons during fictive locomotion Brain Res 149 503 507

The papers are referred to by their Roman numerals in the text

## GENERAL INTRODUCTION

Born into a hostile environment an animal must immediately be able to execute a number of functions in order to survive. Breathing, screaming for food, opening the mouth, swallowing and eliminating body wastes are examples of innate motor behaviours that are specifically and genetically encoded into the nervous system for each species (see Tinbergen 1951, Hinde 1966, Eibl-Eibesfeldt 1970, Bentley & Konishi 1978). Later in life similar innate neural programs control the behaviour of the animal in a number of important activities such as propulsion, reproduction, feeding infants and escaping enemies. It is primarily in the phylogenetically more evolved species that learned motor behaviours play a significant role during the daily life (see Thorpe 1964, Harlow & Harlow 1962, Wilson 1975).

A complete neural explanation of these behaviours involves several factors. A description is needed of the organization of the basic neural networks that are programmed to produce the specific motor task and of the central control (e.g. volition) of these networks. The sensory elements that can initiate activity in the networks and the effector organs that execute the movements should also be identified. These problems can be properly solved in simple motor behaviours such as certain reflexes in mammals or isolated motor functions in invertebrates (see Bentley & Konishi 1978). In the more complex mammalian behaviours the number of neural elements increases to an extreme level.

This summary is limited to the study of the neural control of some innate motor functions in the cat. The studies are focused on the generation of the basic locomotor activity that supports an animal and propels it forward and also on spinal mechanisms involved in the adaptation of the locomotor movements to

the environment. For more extensive reviews on the neural control of the locomotion see Grillner (1976, 1979b), Shik and Orlovsky (1976) and Wetzel & Stuart (1976).

## METHODOLOGICAL CONSIDERATIONS

To understand the neural control of a certain motor behaviour it is important to know the contribution of different parts of the central nervous system as well as the mechanics of the skeleto-muscular system. The organization of the control can be divided into several levels e.g. (i) the mechanisms of the individual neurones (ii) the organization of neurones into networks that generate certain patterns of activity (iii) the function of different receptors and interacting reflex pathways (iv) The final movement depends on the biodynamics of e.g. the limb and of the relevant muscles.

It is almost impossible for the same investigator to master all different levels. We have attempted to study the effect of the integration of the activity from all these levels by means of recordings of the muscular activity, the movements and the forces, but also to explore some neural mechanisms (see above).

Chronic spinal cats were used in papers I-IV. They were subjected to a transection of the spinal cord ( $T_{12}$ ) 1-2 weeks after birth and thereafter nursed and trained carefully to about 6 months of age. The locomotion of the spinal cats on a treadmill was studied in papers I and II. Tactile placing reactions, as described by Bard (1933), were elicited in paper III with strain sensitive devices recording contact and landing of the foot. Stumble corrective reactions elicited during treadmill locomotion were studied in chronic spinal cats (paper IV) and in intact cats (paper V) by tactile stimuli applied to the dorsum of the foot during different phases of the stepcycle.

In paper VI intracellular recording technique was used during fictive locomotion (Edgerton et al 1976). Injection of DOPA and Nialamide in acutely spinalized and curarized cats could initiate rhythmic locomotor activity (Grillner & Zangger

1975) In such preparations it was possible to record from motoneurons and study the mechanisms of phasic gating during ongoing central locomotor activity without any phasic influence from the periphery

**Muscular activity** In papers I-V we used pairs of insulated copper wires ( $\varnothing 0.1$  mm) with 1 mm uninsulated at the tip inserted into desired muscles with hypodermic needles (cf Engberg & Lundberg 1969). Electrode positioning was verified with stimulation between each EMG-electrode and a ground electrode and simultaneous palpation of the contracting muscles. The EMGs were preamplified low (5000 Hz) and high (50 Hz) pass filtered and usually rectified and filtered (see Gottlieb & Agarwal 1970) to study the total envelope of activity. In the later experiment (paper I and II) the rectified and filtered EMG signals were fed via an Analogue Digital Converter (sampling rate 312 Hz) to a Minicomputer (HP 21 MX) for further analysis.

**Movements** The introduction of the cinematography was a great step forward in the study of motor behaviours. The movements of the entire limb and the angular movements of the joints could be analyzed (see Marey 1895, Engberg & Lundberg 1969, Goslow et al 1973 and Philipsson 1905). In the beginning of our studies (papers III and IV and part of I and II) we used a 16 mm film camera with a speed of 64 frames/s. The position of the joints of the hindlimbs was measured on a graphic tablet connected to a desk calculator (HP 9830A) from which the angular movements of the joints could be calculated and plotted as well as the consecutive positions of the limb (stick diagrams). In later studies (papers I, II and V) the movements were recorded by a Selspot System in which infrared light emitting-diodes were glued to the skin over the joints. These diodes were detected by a special camera (156 Hz) from which the x-y coordinates of the diodes could be fed into a minicomputer (HP 21 MX) and stored on magnetic tape. Two cameras allowed simultaneous recording from both hindlimbs (paper II). After standard data treatment (filtering corrections) the angular movements of the joints and the movements of the limb could be directly plotted on a graphic terminal (Tektronix 4010-1).

**Forces** The forces produced by a muscle during ongoing movements have usually been indirectly calculated from movement recordings (film) recordings of the reaction forces (force plate) and with the knowledge of the insertion of the muscles and the mass and center of gravity of each limb segment (Montar 1938 Bernstein 1967 Grillner 1972) Recently it has been possible to attach small force transducers to muscle tendons and directly record the tension from individual muscles (Walsley 1978)

In our work (paper I) we used a forceplate (Kistler 9261A) simply to record the vertical and horizontal forces produced by the limb during the support phase

#### ABBREVIATIONS

The following abbreviations are used Flexion phase F first second and third extension phase  $E_1$   $E_2$  and  $E_3$  M Flexor Digitorum Brevis FDB M Flexor Digitorum Longus FDL; M Gastrocnemius Lateralis LG M Gastrocnemius Medialis MG M Soleus Sol M Plantaris Pl M Quadriceps (Vastus Lateralis) Q M Gluteus Medius GM M Semitendinosus St; M Posterior Biceps PB M Tibialis Anterior TA M Iliopsoas IP M Sartorius Sart M Extensor Digitorum Brevis EDB Electromyography EMG Standard Deviation SD; Standard Error SE

## RESULTS AND DISCUSSIONS

### A SPINAL LOCOMOTION

In 1874 Eichhorst and Kaunyn and Freusberg reported that the lumbar spinal cord isolated from supraspinal structures could evoke rhythmical sequences of flexion and extension of the hind limbs which later was called spinal stepping (see Sherrington 1910) Philippson (1905) analyzed films from spinal stepping in dogs and emphasized the similarity to normal walking. This similarity was however not generally accepted and it was argued that the forelimbs carried the body weight and propelled the animals forward (Magnus 1924 ten Cate 1940). Sherrington (1910) seemed to have accepted spinal stepping as the basis for normal walking but he emphasized the difference between spinal stepping and the "walking" of decerebrate cats. He pointed to the weakness of the extension phase in the spinal animals and suggested that a supraspinal contribution was necessary. Later investigators (Kellog et al 1946 Shurrager & Dykman 1951 ten Cate 1962 1964 Stelzner et al 1975) have improved the walking movements in different animals using different training and manipulative procedures.

Directly after a transection of the spinal cord there is practically no reflex activity caudal to the lesion (spinal shock see Sherrington 1947). Depending on the age and species of the animal it lasted some hours up to several days until locomotor like activity could be induced after the spinal transection (Freusberg 1874 Sherrington 1910 Shurrager & Dykman 1951 Stelzner et al 1975). Noradrenergic precursors (DOPA) or receptor stimulators (Clonidine) could however release spinal stepping or treadmill walking in adult animals immediately after an acute transection of the spinal cord (Grillner 1969 Budakova

1973 Forssberg & Grillner 1973) These walking movements were analyzed in some detail with regard to the temporal pattern of the EMG activity and the adaptation to the speed of the treadmill belt

a) Generation of cyclic locomotor activity in a single hindlimb

Although spinal stepping and spinal walking are well documented in the literature (see above) few attempts have been made to define how complete the locomotor pattern is which the spinal cord can produce without supraspinal influence. In papers I and II we have attempted to do such a study by analyzing the movements, the muscular activity and to some extent the reaction forces during locomotion of chronic spinal cats.

1. Movements Kittens subjected to a transection of the spinal cord at low thoracic level ( $Th_{12}$ ) 1-2 weeks after birth showed alternating flexion and extension movements when lifted in the air 1-2 days after the operation with the spine held vertically. When the animal was placed on a treadmill the alternating movements changed character and the hindlimbs walked with a swing phase in which the foot was lifted in the air and the limb moved forward and with a support phase in which the foot contacted the belt and the limb moved backward in relation to the body. As the kittens grew older the walking improved and based on the angular movement in the hip, knee and ankle joints the swing phase could be divided into a flexion phase (F) during which the foot was lifted with a flexion in all joints and a first extension phase ( $E_1$ ) when the foot was lowered by an extension in knee and ankle during continuing hip flexion. The hip started to extend just prior to foot placement and extended during the whole support phase but due to the movements in knee and ankle the support phase could be divided into a second extension phase ( $E_2$ ) in which knee and ankle flexed due to the sudden load and a third extension phase ( $E_3$ ) in which all joints extended together (see Fig. 1, Philipsson 1905). Similar angular movements have been recorded from locomotion in intact cats (Engberg & Lundberg 1969, Goslow et al 1973). Although the basic movements of the spinal cats were similar to those during normal locomotion there were some deficits. Unless prevented from falling the



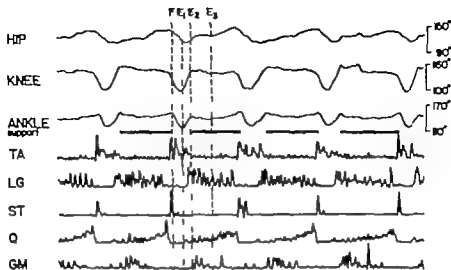


Fig 1 The angular movement of the hip, kn and ankl (at the support) from a chronic spinal cat walking on a treadmill (0.20 m/s) are plotted together with the simultaneously recorded EMGs of TA, LG, ST, Q and GM. The onset of the flexion and extension phases based on the movement of the ankle is indicated with vertical lines. The support phases are presented by schematic blocks. The time calibration is on the bottom: 1 second (1 rg ba) with 200 ms subdivisions (small bars).

hind quarters would fall to one side or the other (equilibrium control). The extension during the end of the support phase ( $E_3$ ) was usually too weak, especially during slow walk, and the knee flexed often before the extensor activity had ceased (see Fig 1). There was also usually a "narrow" walk in which the feet could hook onto each other presumably due to an increased tonus in the adductor muscles of the hip or to a relative weakness in the abductor muscles.

2. Muscular activity. In general, flexor muscles are active during the swing phase and extensor muscles during the support phase, but depending on the joint at which the muscle acts, the activity has a precise onset and termination and a characteristic envelope. Several EMG-studies during locomotion have been per-

formed on intact cats and dogs some reporting both duration and intensity of the locomotor bursts (Engberg 1964 Engberg & Lundberg 1969 Gambarian et al 1971 Tokuriki 1973; dog Smith et al 1977 Malmesley et al 1978 see also paper I) others only the duration (Mentink 1976 dog Rasmussen 1978) Below the pattern of muscular activity during spinal locomotion will be compared to that during normal locomotion (see Fig 2) Unless there are discrepancies in the results from the different EMG-studies of intact locomotion no references will be given

The muscles are referred to as extensor or flexor muscles according to Sherrington's (1910) definition in which extensor muscles counteract gravity during standing and are activated by the crossed extensor reflex and where flexor muscles are participating in the flexor reflex

Extensor muscles of both intact and spinal cats are active approximately from the middle of  $E_1$  (end of swing) until near the end of  $E_3$  (end of support) Engberg & Lundberg (1969) emphasized that the muscles became active before foot placement and that the activity consequently was not induced by reflex effects due to ground contact Extensors of the toes (FDB FDL) and the ankle (LG MG Sol Pl) have an intense onset with an early peak followed by a slow decline and a smooth cessation Corresponding muscles of spinal cats (FDL LG Sol) had the same duration and envelope of activity at corresponding velocities (see Fig 2) The knee extensor (Q) can show two different patterns both seen in intact and in spinal cats Either there can be a continuous burst with a smooth initiation and a successive increase of activity until the very end of  $E_3$  where it ends sharply (see Fig 2) or there can be two separate bursts of which a smaller one occurs during early  $E_1$  and a larger and square shaped one is present during  $E_2$  -  $E_3$  (Engberg & Lundberg 1969 see Fig 4 in paper I) Both patterns could be seen in the same intact or spinal cat the former predominantly at slow walks The hip extensor (GM) had a more symmetric form of activity in both preparations with a smooth onset and termination and a peak during the beginning or middle of the support phase (Fig 2)

Flexor muscles are not as uniformly activated as the extensors The knee flexors (St PB) are activated twice during each step-

## CHRON SPINAL

## INTACT

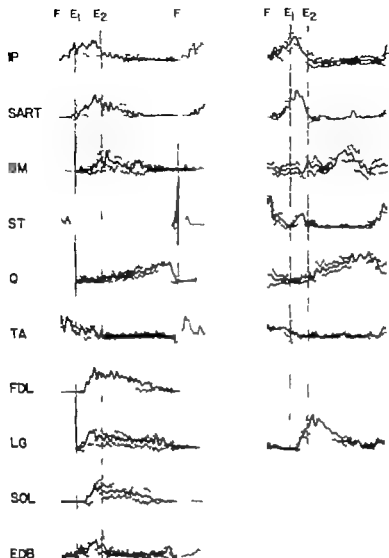


Fig. 2. Averaged EMGs from spinal and intact cats. The rectified and filtered EMGs from the muscles indicated (see Abbreviations) are averaged after normalization of the step cycle duration. The mean is plotted as a continuous line and the standard deviation (SD) as dotted lines. The recordings are from different cats (chron spinal n 2; intact n 2) and from different experimental sessions but are still comparable since all cycle durations are about 800 ms ( $\pm 70$  ms) and as the onset of the averaging is triggered by the movement of the ankle joint (see Methods in paper I). The onset of the flexion and extension phases are marked as vertical lines. The number of averaged locomotor bouts vary between 8 and 12.

cycle in intact animals once with a short and intense burst at the end of  $E_3$  and beginning of  $F$  and a second time with a smaller burst during  $E_1$   $E_2$ . In the spinal cat the first burst was always present while the second burst could be weaker or absent especially at slow walks (see Fig. 2). The second burst was however seen in all spinal cats during faster walk although less prominent than normal. The ankle flexor (TA) has one burst during intact walk that starts somewhat later and is smoother than the first knee flexor burst. In our study (see Fig. 2) as in Wentink (1976) Tokuriki (1973) and Gambarian et al (1971) the plateau like activity outlasted that of the knee flexor and ceased rather suddenly. At the same time the hip flexor activity started to decline with a much slower time course. On the contrary Rasmussen et al (1978) reported TA activity until the beginning of support (longer than hip activity) and Engberg & Lundberg (1969) reported the activity of the ankle flexor to be similar to that of the knee flexor (see however Fig. 22 in Engberg 1964). The hip flexors (IP Sart) have a gradual onset at about the same time as the ankle flexor during intact walk. The activity differs however from that of the ankle flexor and has a pronounced late peak and a longer overall duration (see Fig. Engberg & Lundberg 1969 Tokuriki 1973 Wentink 1976). In the spinal cats the ankle flexor (TA) usually had an intense onset simultaneously with the knee flexor. The hip flexors (IP Sart) started smoother and somewhat later (see Fig. 2). Both hip and ankle flexors were active longer than the knee flexor. The ankle flexor had a successive decrease of activity from the intense onset until the middle of  $E_1$  while the activity of the hip flexors were increased until a peak during mid  $E_1$  and then decreased (see Fig. 2). During intact walking the short toe flexor (EDB) has a short intense burst during  $E_1$  and a residual activity during the larger part of the support phase. A similar pattern was found in the spinal cats (see Fig. 2).

**Conclusion** The EMG-pattern from individual muscles during spinal locomotion shows striking similarities to that of intact walking cats. This implies that the spinal cord with intact afferents can produce a detailed pattern of muscular activity

The specific onset and termination of the activity as well as the characteristic envelope of the locomotor burst is programmed for each individual muscle. There are however some smaller differences as the usual absence of the second St burst at lower speeds. This loss is presumably due to a low excitability level of this part of the network, and not to a loss of the neural components that induce the burst as it could be seen at higher velocities. The more simultaneous activation of knee (St) and ankle flexors (TA) was another difference. It is however noteworthy that a time delay similar to that during normal walk has been found during fictive locomotion (Grillner & Zangger 1979).

3 Force During the support phase the extensor muscles shall support the body and propel it forward. The extensors of the hindlimbs of the spinal cats could carry the weight of the hindquarters during locomotion, although the cats needed assistance to maintain equilibrium (see Fig. 6 in paper I). The knee did however not manage to extend during the end of  $E_3$  when the maximal force was required (see below) but yielded under the load (see above). This yield could be due to either insufficient muscular force or to a required torque that was higher than normal. The forwardly directed force from the hindlimbs mainly required from the hip extensors was clearly too weak to bring the animals forward alone. It was necessary to extend the hips to initiate walk unless an unspecific stimulus was used (see below). But also when the cats were stimulated e.g. squeezing the tail the forward force was very weak (see Fig. 6 in paper I).

The force required from the extensor muscles during a support phase in normal cats has been calculated from combined film and force plate recordings (Huxter 1938, Grillner 1972) and recently also recorded from force transducers attached to muscle tendons (Wassley et al. 1978). In the ankle and the hip the maximal torque is required in the beginning of the support while in the knee it is needed in the end (Huxter 1938, Zoller et al. unpubl.). The relative envelope of the locomotor bursts in spinal as well as in the intact cats corresponds surprisingly well to the required forces at the different joints during the different phases of support (as calculated in intact cats) (see Fig. 2).

The flexor muscles have to both stop and reverse the direction of the movement of the limb. The hip muscles have an important function in moving the limb forward during the largest part of the swing phase. The main task of the more distal flexors beside stopping the extension is to flex the limb in the beginning of swing in order to bring the mass of the limb closer to the pivot point. It is therefore functional with the early and short activity in the distal flexors and the later main activity in the hip flexors (cf. Engberg & Lundberg 1969). The short burst of the knee flexors (St or PB) is presumably due to the double function of the muscles who also extend the hip. They would counteract the flexion if active in later parts of the swing phase when the hip is flexed (Grillner 1975).

4 Speed adjustments When the speed of locomotion increases in intact cats the number of stepcycles per second increases markedly. At the same time there is at least at lower speeds a substantial increase of the support length i.e. the distance the animal moves during each support phase (see Grillner 1975 paper I). The reduction of the cycle duration is largely due to a shortening of the support phase with only a small reduction of the swing phase (mainly E<sub>1</sub>) (Goslow et al. 1973; Halbertsma 1976). The locomotion of the spinal cats followed within some limits the speed of the treadmill belt. Similar adjustments of the support length and of the phases did occur as during intact locomotion at faster walks. The duration of the extensor bursts shortened in parallel with the reduction of the support phase with maintained total muscular activity due to an increased overall intensity. A prolonged and enhanced flexor activity succeeded to break the enhanced extension and to slightly shorten the swing phase (see Fig. 2 in paper I). Hence there are appropriate speed adjustments of the spinal mechanisms that produce the locomotor activity.

5 Central generation Throughout the animal kingdom it has been shown that a variety of innate motor behaviours such as breathing (Bradley et al. 1975), mastication and feeding (Dellow & Lund 1971; Miller 1972; Maynard 1972; Selverston 1976) and locomotion (Wilson 1961; Miersma & Ikeda 1964; Székely et al.

1969 Pearson 1972 Grillner & Zangger 1975 Grillner et al 1976) are coordinated by means of central neural networks. It has been demonstrated that a significant part of the networks that generate locomotion in the cat are located in the spinal cord as spinal deafferented (Brown 1911 1914) or curarized preparations (Jankowska et al 1967a b Grillner & Zangger 1974 1979 Viala & Buser 1969 rabbit Vidal et al 1979 rabbit) can produce alternating flexor and extensor bursts with some retained temporal specificity between different type of muscles (Grillner & Zangger 1979 see also Fig 2 3 in Vidal et al 1979). Paper I demonstrates that spinal cats with intact afferents can produce a replica of the intact locomotion and studies on decerebrate and decorticate cats that the detailed timing of the different locomotor bursts can be generated without phasic feedback related to the movements (Grillner & Zangger 1975 (and unpubl.) Perret & Cabelguen 1975).

6 Peripheral control of the locomotor generator. The fact that locomotion was induced when a spinal cat was placed on a moving treadmill belt and adjusted to the velocity of the belt (see above) implies that peripheral influences are exerted on the locomotor generator (paper I). Sherrington (1910) noted that spinal stepping occurred when the animals were held with vertical spine and he found that hip extension was sufficient to initiate stepping while passive extension of ankle or knee alone or together did not suffice. Orlovsky (1972) showed during on going locomotion in mesencephalic cats that an arrest or an acceleration of the hip flexion prolonged or reduced the duration of flexion of the entire limb.

The locomotion of the hindlimbs of the spinal cats was initiated by extension of the hip both when the animal was held over a treadmill with the feet on the belt and when the animal was standing on the ground and started to walk with the forelimbs (Budakova 1973 Forssberg & Grillner 1973 paper I). If during treadmill locomotion one hindlimb was stopped during the support phase the extensor activity was maintained until the limb was brought backward to the position of the hip where flexion usually occurs during normal locomotion. At this position the extensor activity suddenly ceased and the limb briskly flexed.

(Rossignol et al 1976 Grillner & Rossignol 1978) Similarly flexed hip position was found to promote extensor activity during fictive locomotion (Andersson et al 1978) These effects where the position of the hip influences the locomotor generator to switch from extensor to flexor activity (Rossignol et al 1976 Grillner & Rossignol 1978 Andersson et al 1978) or to initiate extensor activity (Andersson et al 1978 Grillner 1979a cf Orlovsky 1972) has been regarded as a position dependent negative feedback (Grillner 1979a) Andersson et al (1978) have however also described a directional sensitive mechanism in which small movements of the hip entrained the rhythmic activity during fictive locomotion (Grillner 1979a; cf Grillner & Wallén 1977)

The position dependent control regulating the amplitude of hip extension during locomotion, will control the duration of the support phase during different speeds. But what induces the enhancement and prolongation of the flexor bursts that occur at increasing velocity (see paper I)? It seems probable that events in the ipsilateral limb occurring prior to the flexor activity would be the determining factor such as the duration of the extensor activity or the velocity of extension. It is also possible that an increased rate of unspecific stimuli that are known to excite the locomotor activity (cf Grillner 1975 Grillner & Zangger 1979 paper I cf Sherrington 1910) could play some role in the speed adjustments.

Pearson & Duysens (1976) found that an extensive load applied to the ankle extensor muscles could prevent the switch from extensor to flexor activity. During normal locomotion the torque around the ankle decreases at the end of support (see force above). An increased load on the ankle extensors would imply that the limb is not brought backward or that there is an increased load on the limb (cf Grillner 1979a). In both situations it would be meaningful with a prolonged support and a delayed swing.

#### b) Interlimb coordination

During normal quadrupedal locomotion the movement of each limb has to be coordinated with the movement of the other three limbs. An animal can select among a number of patterns depend



1969 Pearson 1972 Grillner & Zangger 1975 Grillner et al 1976) are coordinated by means of central neural networks. It has been demonstrated that a significant part of the networks that generate locomotion in the cat are located in the spinal cord as spinal deafferented (Brown 1911 1914) or curarized preparations (Jankowska et al 1967a & Grillner & Zangger 1974 1979 Viala & Buser 1969 rabbit Vidal et al 1979 rabbit) can produce alternating flexor and extensor bursts with some retained temporal specificity between different types of muscles (Grillner & Zangger 1979 see also Fig 2 3 in Vidal et al 1979). Paper I demonstrates that spinal cats with intact afferents can produce a replica of the intact locomotion and studies on decerebrate and decorticate cats that the detailed timing of the different locomotor bursts can be generated without phasic feedback related to the movements (Grillner & Zangger 1975 (and unpubl.) Perret & Cabelguen 1975).

6 Peripheral control of the locomotor generator. The fact that locomotion was induced when a spinal cat was placed on a moving treadmill belt and adjusted to the velocity of the belt (see above) implies that peripheral influences are exerted on the locomotor generator (paper I). Sherrington (1910) noted that spinal stepping occurred when the animals were held with vertical spine and he found that hip extension was sufficient to initiate stepping while passive extension of ankle or knee alone or together did not suffice. Orlovsky (1972) showed during on going locomotion in mesencephalic cats that an arrest or an acceleration of the hip flexion prolonged or reduced the duration of flexion of the entire limb.

The locomotion of the hindlimbs of the spinal cats was initiated by extension of the hip both when the animal was held over a treadmill with the feet on the belt and when the animal was standing on the ground and started to walk with the forelimbs (Budakova 1973 Forssberg & Grillner 1973 paper I). If during treadmill locomotion one hindlimb was stopped during the support phase the extensor activity was maintained until the limb was brought backward to the position of the hip where flexion usually occurs during normal locomotion. At this position the extensor activity suddenly ceased and the limb briskly flexed.

between the belts the fast limb could perform a multiple of steps (2-4) during one stepcycle of the slow limb (Kulagin & Shik 1970 paper II cf von Holst 1939) The duration of the stepcycles of the fast limb as well as the flexion and extension phases differed markedly depending on the phase of locomotor activity of the other limb (paper II)

These findings imply that there is a potent interaction between the locomotor generators (see von Holst 1939 Arshavskiy et al 1965 Shik & Orlovsky 1965 Gelfand et al 1971 Kulagin & Shik 1970 Stein 1976 Grillner 1975 1979b Grillner & Zangger 1979) that can influence each other particularly during the swing phase to generate the same or multiple rhythms though the peripheral influences differ Grillner and Zangger (1979) showed from experiments with fictive locomotion that the locomotor activity on the two sides were properly coordinated which implies a pure interaction between the generators It is however important to realize that the peripheral influence on one side also directly may influence the contralateral generator It can thus not be taken for granted that the effects are only due to interaction between the two independent generators

Disregarding the detailed neural mechanism of the interaction an interesting implication is that the brain does actually not need to concern itself with adjustments of the hindlimbs when the animal is turning It would be sufficient for the front part to turn and the hindlimbs would by the diverging peripheral influences from the two hindlimbs and by the interaction of the generators make the appropriate changes (paper II cf Grillner 1979b)

### c) Concluding remarks

Neural networks within the spinal cord with intact reflex interaction can generate locomotion in the chronic spinal cat The spinal mechanisms can even reproduce subtle details of walking movements and of signals to the muscles (EMGs) of the intact walking cat A central locomotor generator produces specific locomotor activity Due to peripheral influence the generator adjusts the locomotor activity to different speeds The activity of the two limbs is properly coordinated by mutual interaction of the two locomotor generators With such a comp-

lete organization in the spinal cord it would be sufficient for supraspinal centers simply to control the excitability level of the neural circuits (cf Grillner 1975). An increased tonic facilitation could increase the activity of the generators which alone could make the required adjustments. Such spinal circuits may indeed operate also in the normal and adult cat as locomotion can be induced in acute spinal cats after injection of DOPA or Clonidine (Jankowska et al 1967a, b Grillner 1969 Budakova 1973 Forssberg & Grillner 1973).

## B PLACING STEPPING AND HOPPING REACTIONS

### a) The reactions

Magnus (1924) Rademaker (1931) and Bard (1933) described several placing and hopping reactions that were assumed to aid animals in placing their feet. Visual placing occurred when the head approached an object and consisted of a flexion of the limb and a subsequent placing of the foot on the top of the object. Exteroceptive placing could be elicited from the chin the vibrissae the tail or the dorsum of the foot. If an animal was held in the air with its limbs hanging a light contact of the dorsum of the foot with the edge of a table would elicit a tactile placing reaction in which the foot was immediately withdrawn and accurately placed sole down close to the edge of the table (Bard 1933). Touching the medial or lateral aspect of the foot resulted in a placement in a more medial or lateral position (see Amassian et al 1972). A stimulus to one foot could induce bilateral reactions (Bard 1938). A more extensive contact that would include activation of proprioceptors elicited a reaction similar to tactile placing. If the body of a standing animal was displaced forward backward or sideways in relation to the feet a new and more appropriate limb position could be achieved by making a step (hop) in a direction that resulted in a more stable stance. If all limbs (or two) were free it would execute consecutive steps. If standing on one limb (other limbs suspended) it could hop by a flexion and a subsequent extension in the direction of the displacement (Rademaker 1931 Bard 1933).

A hop correction for a forward movement is thus very similar to the placing evoked from a displacement of the dorsum of the foot as proprioceptors from the proximal part of the limb (hip) are contributing to both reactions (Rademaker and Hoogenwerf 1930 Bard 1938). Similar responses may thus be evoked by different receptor systems even if they are modifiable in relation to the site of the visual or exteroceptive stimuli or the direction of the displacement.

Tactile placing has been studied more than the other reactions. The flexor muscles become activated 20-40 ms after foot contact and the succeeding extensor activity evokes placing of the foot 180-550 ms after contact (Amassian et al 1972, Morrissell and Lundberg in Lundberg 1973)

#### b) Basic organization in the spinal cord

Stepping, hopping, proprioceptive and tactile placing reactions could all be elicited in animals subjected to a transection of the spinal cord at lower thoracic level (Forsberg et al in Grillner 1973, paper III, Steizner et al 1975, rat, see also Lundberg 1973). A very light touch by a fine brush only deflecting the hairs on the dorsum was sufficient to elicit a tactile placing reaction in the hindlimbs which in all aspects corresponded to Bard's original description (1933). Also lateral or medial placing (see above) could be evoked (paper III). In contrast to what was thought earlier (Bard 1933, 1938, Amassian et al 1972) these corrective reactions of the hindlimbs can thus be generated by spinal reflex networks.

The striking similarity of the different reactions suggest that the same neuronal network is used although it becomes activated by different types of receptors. A seemingly less likely alternative is that each different receptor system should have its private placing network. The flexion-extension movement resembles a single step (see Bogen and Campbell 1962). The basic locomotor activity (flexion-extension) is generated by a central spinal network which can be influenced from the periphery for instance by hip movements (see spinal locomotion above). It is possible that different correcting reactions at least in part utilize the central locomotor network to induce a step (paper I).

The hip extension that may initiate locomotion on a moving treadmill is the same kind of stimulus that can evoke a hopping or stepping reaction in the forward direction. Spinal cats can not only produce a backward hop or step but also actually walk backwards on a treadmill (Forsberg, Grillner & Halbertsma, unpublished). Furthermore tactile placing can generally be evoked with lower threshold and brisker reactions just after the chronic spinal cats have been walking (paper III). What about the medial/lateral placing reactions? A basic flexion-extension movement

is clearly involved that may be caused by the locomotor generator (see preceding paragraph) but a direction specific reflex effect must also be added. Such an effect could simply be due to additional excitation to abductor (lateral placing) or adductor (medial placing) motoneurons that in any event are activated by the locomotor generator.

### c) Supraspinal control

Bard and co-workers performed a series of studies in different species on placing and hopping reactions in the beginning of the 1930's which have influenced the literature ever since. Hopping and proprioceptive placing but not tactile placing could be elicited in reptiles (alligator lizard) (Bard et al 1932 see Bard 1938). Both tactile and proprioceptive reactions could be evoked in rat or rabbit but tactile placing was not as well developed as in the cat or the monkey. After decortication it was abolished in all species while the proprioceptive reactions remained in rat, rabbit and in the reptiles (Bard et al 1932, Bard 1933, Brooks 1933, Brooks & Woolsey 1936, Woolsey & Bard 1936, Bard 1938). Tactile placing of the cat was further shown to be dependent on a restricted area of the contralateral sensorimotor cortex and was depressed if this area was removed but remained when the entire cortex except this part was taken away (Bard 1933). On the basis of these experiments it was suggested (see Bard 1933, 1938) (i) that the neural mechanisms of the reactions migrated to higher levels during phylogeny, (ii) that the tactile reactions were more advanced than the proprioceptive ones and organized on a higher level and (iii) that a localized area of cortex was an essential part of the tactile reactions in higher animals (cat, monkey).

In experiments on monkeys Bard found that hopping reactions depressed after a contralateral ablation of the cerebral cortex reappeared when the ipsilateral cortex was also removed. After this finding he modified his suggestion concerning the proprioceptive reactions and pointed to the possibility that subcortical centers could induce the reactions although a tonic control was exerted from cortex (suggestion of McCulloch in Bard 1938). Later investigators also found a reappearance of tactile placing after an ipsilateral decortication following a contralateral one (Denny-Brown & Chambers 1958, Bogen & Campbell 1962).

Tactile reactions have also been elicited in extensively neocorticated cats and in acutely decerebrated cats after an injection of d amphetamin (Maling & Acheson 1946 Meyer et al 1963) and in chronic spinal animals (paper III Stelzner et al 1975)

Since a spinal network has been shown to generate tactile placing in the hindlimbs (see above) the original view suggesting long loop reflex (including even cortex) cannot be the only mechanism that produces the reaction (see Fig 3 alternative 1)

Independently of its mechanism the depression of the reactions following a contralateral ablation of the sensori-motor cortex (Bard 1933) depends on the pyramidal tract (corticospinal tract) as a depression occurs also after a pyramidotomy (Tower 1940 Gilman & Marco 1971) The reappearance of the reactions following an ipsilateral hemi decortication (Bard 1938 Denny Brown & Chambers 1958 Bogen & Campbell 1962) must result from a net facilitation of the circuits that are responsible in the decorticated state They may be spinal but additional supra spinal loops cannot be excluded (see below)

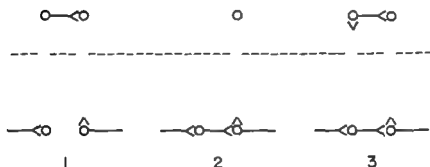


Fig 3 Three theoretically different organizations of the neural reflex network that may produce tactile placing. One interneurone in the diagram may represent a chain. 1) A long loop reflex mediated via supraspinal structures. 2) A spinal basic network tonically influenced from higher level. 3) A long loop reflex superimposed on the spinal reflex network.

The ipsilateral Nucleus Interpositus also seems to facilitate tactile placing (Snider 1940 Chambers 1953 Chambers & Sprague 1955) presumably via the red nucleus (cf Pompeiano 1967 Amassian et al 1972) The intermediate part of the cerebellar anterior lobe has an inhibitory influence on Nucleus Interpositus and a selective lesion enhances placing (Pompeiano 1967) Ipsilateral lesions of Nucleus Reticularis Lateralis or an ipsilateral denervation of the neck (C<sub>1</sub>-C<sub>3</sub>) abolish the placing reactions presumably due to effects on nucleus interpositus (Corvaja et al 1977 Manzoni et al 1979) Information from several central nervous structures can thus via cerebellar circuits influence the performance of the placing reactions

Lesions of an uncrossed pathway in the mediodorsal part of the lateral funicle abolishes tactile placing (Lundberg & Morrissell 1960) Activity related to placing reactions occurs in the ventrolateral nucleus of the contralateral thalamus (Amassian et al 1972 Hession & Smith 1973 1974) Finally an ablation of the post central gyrus abolishes placing of the contralateral limb as well as crossed placing of the untouched limb (Bard 1938 Lumley 1973) Theoretically the supraspinal control can be exerted in two different ways (i) by tonic facilitation or inhibition (alternative 2 in Fig 3), or (ii) by long loop reflexes superimposed on the spinal reflex networks (alternative 3 in Fig 3) The ascending activity could constitute an afferent link in a long loop (alternative 3) but the depression of the reactions seen after the lesions can as well be due to indirect tonic effects on presumed cortical or subcortical centers that control the spinal networks (i.e. alternative 2) There are no possibility at present to decide between the remaining alternatives (2 or 3 in Fig 3) The supraspinal centers in intact cats can nevertheless control the reaction and depress it when it is not useful (see Amassian et al 1972)

It is noteworthy that several authors influenced by Bard's original reports still discuss placing reactions as cortical reflexes (Henneman 1974) or as learned responses dependent on the integrity of motor cortex (Roberts 1978; pp 173 and p 296) or use the reactions to study higher level control of movement (Amassian et al 1972 1977) or development of the corticospinal tract (Donatelli 1978) although it has long been known that sub-



cortical centers can generate the reactions (Bard 1938 Maling & Acheson 1946 Denny Brown & Chambers 1958 Bogen & Campbell 1962 Meyer et al 1963 Stelzner et al 1975 paper III)

d) Development of the control of the tactile placing reaction

Tactile placing in the rat develops 7-18 days after birth at the same time as the pyramidal tract grows into the spinal cord (Tang 1935 Hicks & D'Amato 1975 Donatelle 1978) When a hemispherectomy was performed at birth the tactile placing developed as usual (from the end of the first week) until around the 17th day when the responses ceased contralateral to the lesion. If however the motor cortex was removed bilaterally at birth no later impairment occurred (see Hicks & D'Amato 1975). A mid-thoracic lesion of the rat spinal cord prior to the 15th postnatal day neither disturbed tactile placing nor the existing locomotion while it later induced severe disturbances and could abolish the reactions altogether (Stelzner et al 1975 Weber & Stelzner 1977 see also Bignall 1974)

After a unilateral removal of sensorimotor cortex in kittens before the fourth postnatal week tactile placing first returned but was subsequently lost at one to two months of age (Amassian & Ross 1973). Cooling of the cortex (kittens) had no effect during the first postnatal week but abolished placing after the end of the second week (Amassian 1976)

There is thus an initial period during which a blockage of the supraspinal control does not affect the performance of the tactile placing reactions or locomotion. During this period spinal networks must be responsible to a significant degree. Afterwards these networks somehow become dependent on supraspinal structures. The actual mechanism is unknown. It should be noted however that the motor reactions can be activated in adult animals after lesions at different levels and that the networks thus still are intact (Maling & Acheson 1946 Meyer et al 1963 see also Grillner 1969 Budakova 1973 Forssberg & Grillner 1973)

e) Modifiability of placing reactions during different conditions

If an animal standing on all four limbs is mechanically stimulated on the foot dorsum postural adjustments occur first in which the contra- and the homolateral limbs increase the sup

port while the stimulated and the diagonal limbs decrease their supporting force. There is a small early activation of the flexors of the stimulated limb but only after the postural adjustments (mean 480 ms) a real flexion and a subsequent placing occur (Massion & Smith 1973, 1974). The same kind of stimulus evokes in a cat with free pendant limbs instead fast ipsi- and contralateral flexions (80-200 ms) (see above). A short and weak electrical pulse (1 mA, 1 ms) applied to the foot dorsum elicits a flexor burst with a latency (25 ms) corresponding to that during tactile placing. If the animal stands however this reflex burst is depressed and instead a faster burst with shorter latency emerges (paper V, Fig. 9). These findings imply that there are several alternative programs that may be activated by the same tactile stimulus. Different short latency pathways may be selected and the generation of flexion may even be delayed until appropriate postural adjustments have been made and the contralateral limb may be extended or flexed. The program utilized is influenced by the ongoing motor activity. In the next chapter it will be shown that the locomotor activity to a large extent can control the reflex pathways from the foot dorsum.

## C STUMBLING CORRECTIVE REACTION

### a) The reaction

Cats are able to avoid most obstacles during forward locomotion but they need to deal with unexpected perturbations. If an obstacle impedes the forward movement of the foot during locomotion a short latency reflex activation of the flexor muscles occurs and as a result the limb is briskly flexed and lifted above the obstacle (see Fig. 4). During early swing an enhanced

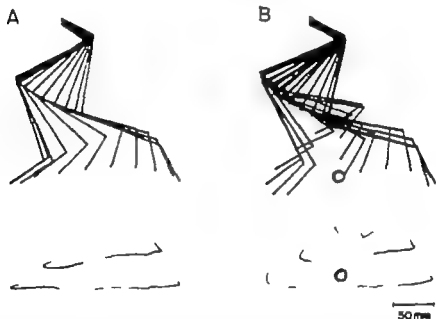


Fig. 4 Movement analysis in an intact cat walking on the treadmill of A: a normal swing phase and of B: a stumble corrective reaction elicited by a tick inserted in front of the limb during the swing phase. The movements are recorded by a 81 port system from which the position of the hip, pelvis (iliac crest), knee, ankle and tarsometatarsal joints are obtained (see Methods in paper V). At the top, the movement of the left limb is displayed with stick diagrams in which straight lines between the joints (see above) represent consecutive limb positions at 30 ms intervals (limb moves from right to left). Below the stick diagrams, the trajectory of the paw (tarsometatarsal joint) and ankle are plotted with 6 ms interval in relation to the pelvis. The pelvis is fixed in the horizontal direction in both stick diagrams and trajectory.

flexion is evoked but later when the knee and ankle have started to extend a new flexion begins. On the other hand when the dorsum of the foot is similarly stimulated during the support phase no flexor responses appear but instead a reflex burst preceded by inhibition is evoked in the extensors and a very brisk flexion is induced in the following swing phase. In the spinal cat the extensor excitation is strong enough to induce an enhanced extension which pushes the animal further forward upward. The reflex responses are thus phasically modified to produce reactions adapted to compensate for disturbances in accordance with instantaneous and dynamically changing conditions of the limb in different phases of the stepcycle. It has been called the stumbling corrective reaction (papers IV and V).

#### b) Receptors and receptive fields

If the skin is anaesthetized before contact the foot instead of being briskly lifted over the obstacle continues to push against it and is then pulled over the obstacle (Forssberg et al. 1975, Prochazka 1978, paper IV and V). Thus skin receptors (touch) are one main source for the reaction. Proprioceptors can, however, induce activity that may stabilize the joints during the reaction (see Prochazka 1978). Different types of stimulation of the foot dorsum consisting of an airpuff (hair receptors), a mechanical contact (including mechano receptors), a weak (1 mA, 1 ms) or an intense electrical pulse (10 mA, 10 ms, including nociceptors) all evoke a short latency flexor response during the swing phase, depressed during the support phase and similar stumbling corrective reactions (paper V).

Cutaneous receptors located more proximal on the limb can elicit a reaction in which the limb is lifted higher than after foot contact. Even a stimulus to the hairs on the abdomen evokes a similar reaction. This is meaningful as a high obstacle that impedes locomotion will first touch the belly leading to an enhanced flexion even before the limb has hit the obstacle (paper V).

#### c) Reflex pathways

Five different reflex pathways involved in the reaction have been identified by an electrical stimulus (1 mA, 1 ms)

applied to the dorsum of the foot during treadmill locomotion (paper V) There is one early reflex in the flexor muscle (St) with a latency of about 10 ms and one later occurring about 25 ms after the stimulus Two corresponding bursts can be found in the extensor muscles one at about 10 ms (LG) and a second with a longer latency (25-50 ms) In addition an inhibition starts around 10 ms after stimulation with a duration varying in different extensor muscles until 25-50 ms after stimulation The same kind of tactile stimulus can evoke independent but not simultaneous activity in all 6 pathways (see phasic gating below)

The early flexor excitation and the extensor inhibition correspond to the flexor reflex effects (Sherrington 1910 see Creed et al 1932) This reflex can be elicited by all types of afferents except group I fibres (Eccles & Lundberg 1959a, b) It is possible that the corresponding reflex responses of the stumble corrective reaction are elicited by the same pathways Also in the flexor reflex the flexion in different joints could be modified depending on the site of the stimulus i.e. the local sign (Creed et al 1932) Earlier Sherrington noticed that a stimulus that evoked the flexor reflex could elicit a flexor inhibition or an extensor excitation under other conditions (Sherrington & Sowton 1911a, b Cooper et al 1927 see also Holmqvist & Lundberg 1961) Intracellular recordings from triceps surae motoneurons revealed that a cutaneous stimulation usually evoked a combined EPSP/IPSP of which excitation dominated in fast motor units and inhibition in slow ones (Burke et al 1970) The EPSP corresponds to the early extensor excitation of the stumble corrective reaction during the swing phase and the IPSP to the inhibition during the support phase No flexor inhibition could be evoked by paw stimulation during walking in any preparation as recorded by EMG

The latencies of the later bursts are consistent with a transmission to supraspinal centers and back (Shimamura & Livingston 1963) The responses occurred however in spinal animals and can thus be induced by spinal networks (see paper V) Hagbarth (1952) described how cutaneous stimuli could evoke specific excitatory and inhibitory effects depending on the stimulus site in spinal cats with latencies corresponding to both the earlier and the later reflex bursts of the stumble corrective

reaction. The extensor muscles were inhibited from most parts of the limb but excited from a skin area localized mainly over the muscle itself. The flexor muscles were usually excited except from the area above the antagonistic extensor muscle.

The stumble corrective reaction could be elicited from different types of receptors on the dorsum of the paw with only minor differences of the reactions. Hagbarth (1952) showed that the responses after skin stimulation was the same either small or large fibres were activated. In both studies however as in the flexor reflex (local sign) the responses depended on the stimulus site.

It is tempting to relate the late flexor response of the stumbling corrective reaction to the tactile placing as the same type of stimuli evokes both reactions and the latency of the tactile placing is the same as the late flexor burst i.e. 25-30 ms (cf. Aamassian et al. 1972; Norrsell & Lundberg in Lundberg 1973). It was previously suggested that tactile placing consists of a step generated by the locomotor generator and a directional specific reflex that induces the step and directs it in the lateral or medial direction (see above). The initial flexor activity (25-30 ms) can then either be the result of a reflex activity or constitute the beginning of the flexor locomotor burst. The fact that the latency is similar during ongoing locomotion may suggest that there is first a reflex activation of the flexors which later can be maintained by the locomotor generator which is initiated by the reflex. The response evoked in the stumble corrective reaction would then reflect the reflex part of the tactile placing superimposed on the already ongoing locomotion with a specificity depending mainly on the stimulus site.

The intensity and the duration of the stimulus can however also influence the response. A weak and short stimulus evoked much smaller reactions than a longer. During the support phase a weak but long (1 mA train 40 ms) or an intense and short (10 mA 10 ms) electrical stimulus on the foot dorsum evoked only the characteristic extensor responses. In contrast an intense and long (10 mA; train 40 ms) stimulus evoked a concomitant flexor response (i.e. a flexor reflex broke through).

V) The transmission in the reflex pathways to the FI thus be inhibited to an extent that prevents effects

trains in large afferents or short pulses in smaller ones (nociceptive). A long spike train including small nociceptive afferents will however elicit a flexor reflex. The difference can either be due to a summation of the compound signals from the different afferents or to a more specific effect from the small nociceptive fibres with a strong projection to the flexors. This latter response clearly does not belong to the stumbling corrective reaction (paper V).

#### d) Differences of responses in intact and chronic spinal cats

The clearest difference of the stumbling corrective reaction between intact and spinal cats was the amplitude of the reflex responses. During spinal locomotion a small extensor inhibition and a large excitation resulted in an enhanced extension during the support while an increased inhibition and a suppressed excitation canceled each other in the intact cat. There were smaller differences in the early excitation of both extensor and flexor muscles while the late flexor response was larger in the intact preparation (see paper V Table 1).

Many descending pathways such as cortico- rubro- and different reticulospinal pathways are known to influence the transmission of spinal cutaneous reflexes (Holmqvist & Lundberg 1961, Lundberg & Voorhoeve 1962, Engberg 1964, Engberg et al 1968, Hongo et al 1969, Burke et al 1973, Grillner & Shik 1973). Some of these pathways are phasically active during locomotion (Orlovsky 1970, 1972) and it may be expected that the degree of excitability in the reflex arcs can vary between the intact and spinal conditions. The difference of the reflex bursts can be due both to phasic and tonic changes of the transmission in the relevant spinal pathways and also to complementary supra spinal loops (see Fig. 3).

#### e) Phasic gating

The stumble corrective reaction is phasically adapted to produce functionally meaningful reactions in each phase of the stepcycle. The phasic modulation of the four excitatory reflex responses is shown in Fig. 11 in which the amplitude of the rectified and filtered EMG-responses is plotted in the different phases of the stepcycle in an intact walking cat. The early and

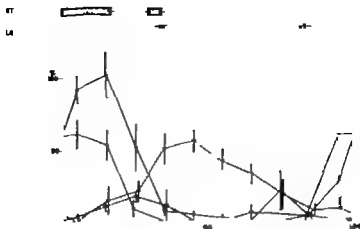


Fig 5 Phase dependence of the reflex response in St and LG during treadmill locomotion in an intact cat. The paw is electrically stimulated (2 mA, 1 ms). The mean amplitude of rectified and filtered EMG responses ( $\pm$ SE) is calculated for each 10th of the step cycle expressed as a percentage of the mean maximal of 10 normal locomotor bursts and plotted versus the phase of time-lation in the step cycle. The step cycles are normalized to the onset of the main activity in St. The schematic EMGs present 10 normal locomotor bursts (SD). The symbol for St and LG are used in the graph in which open symbol indicates the early flexor responses (ca 10 ms) and the filled the later response (25-50 ms).

the late flexor responses but also the early extensor response (though small) are largest during the flexor period while the late extensor response is largest during the extensor period (see also Fig 6A). The fifth reflex response is the extensor inhibition, is present when the extensor muscles are active. Whether an inhibition occurs also during other periods cannot be investigated by EMG-recordings.

The relative amplitude of the responses is phasically modulated in a qualitatively similar way in walking spinal cats (see Fig 6B and above) which implies that a spinal mechanism related to locomotion would act as a gain control. The simplest explanation would be that all reflex pathways are continuously open and that the phasic changes of the motoneuronal membrane potential that occurs in each step cycle (Edgerton et al 1976) would determine whether or not the synaptic potentials will be sufficient to reach the threshold to discharge the motoneurons. Such a mechanism cannot be responsible for all the effects as e.g. the early extensor burst which is largest during flexor



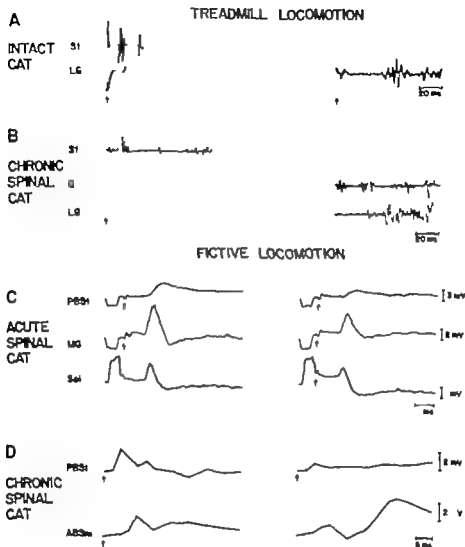


Fig. 8 Reflex responses evoked by an electrical pulse (1 mA, 0.5 ms, 5 mA, 5 ms) during different periods of locomotor activity in different preparations. The flexor period is defined as the interval from the onset of flexor activity (St) to the onset of extensor activity and the extensor period as occupying the remaining part of the locomotor cycle. In A and B single reflex responses are recorded in the muscle by EMG. In C and D the responses are intracellularly recorded from the motoneurons. In C the responses are averaged on an HP 21 MX computer (n=12-28) and in D calculated from single graph recordings (n=18-37). The stimulus is indicated by an arrow. In C a conductance pulse (constant current) is injected through the microelectrode prior to the stimulus.

activity (see Fig. A C D) or the two reflex responses to the knee flexor (St) which both are large during the first locomotor burst but depressed during the second smaller one (see Fig. 5). A more complex mechanism would be a phasically changed efficiency of the transmission in the reflex pathways at a premotoneuronal level. Such a phasic gain control can be exerted either (i) by the spinal locomotor generator that would influence the elements of the reflex pathways concomitant to its efferent outflow to the motoneurons or (ii) by interaction from peripheral afferents that are phasically activated by the locomotor movements (see Fig. 10 in paper IV).

Curarized low spinal cats performing fictive locomotion (Grillner & Zangger 1974, 1975, 1979) were used to explore the neural mechanism of this gating. In these preparations rhythmic activity related to locomotion is elicited by means of noradren-ergic precursors or receptor stimulators. The neuronal activity can be recorded in peripheral nerve filaments and of course with micro electrode techniques. One advantage for our purpose is that the central locomotor generator is active while the movements are blocked. Electrical stimuli applied to the dorsum of the paw which in the walking cats evoked stumble corrective reactions evoked in the motoneurons several combinations of excitatory (EPSPs) and inhibitory postsynaptic potentials (IPSPs). Usually there was an EPSP in flexor motoneurons, 7-8 ms after the stimulus and a corresponding EPSP followed by an IPSP in extensor motoneurons (see Fig. 6C, see also Burke et al. 1970). Twenty two flexor and 37 extensor motoneurons from 12 cats were recorded (paper VI and unpubl.). The amplitude of the EPSPs of both flexor and extensor motoneurons was usually largest during flexor activity and they were significantly larger in 9 flexor and 16 extensor motoneurons ( $P < 0.05$ ; Student's t test see Fig. 6C, Fig. 7 and also Edgerton et al. 1976, Andersson et al. 1977). The amplitude of the IPSP in the extensor motoneurons was often larger during extensor activity although it varied more (see Fig. 6C, Sol.). Only occasionally late extensor EPSPs were evoked by paw stimulation. A late reflex EPSP could be evoked in some flexor motoneurons but only during the early period of flexor locomotor activity (see Fig. 1.3 in paper VI).

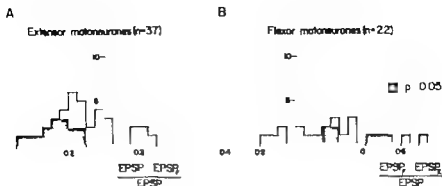


Fig. 7 The relative modulation of the early EPSP in acutely pinalized cats performing fictive locomotion. The difference of the averaged amplitude (Fig. 6C) between the flexor and extensor period (see Fig. 6) is normalized and plotted as histograms for extensor and flexor motoneurons (see Results in the figure). Shaded units indicate motoneurons in which the responses are significantly ( $p < 0.05$ ) different in the two periods (Student's *t*-test).

Intracellular recordings from a chronic spinal cat performing fictive locomotion showed however also a second large EPSP with a latency of 20–40 ms in the extensor motoneurons. This EPSP was much larger during the extensor activity ( $p < 0.001$ ; Student's *t*-test) while the early extensor and the flexor EPSP were larger ( $p < 0.01$ ) during flexor activity (see Fig. 6D). Late EPSPs were not seen in flexor motoneurons of the chronic spinal cat.

Theoretically the observed changes in PSP amplitude could be attributed to pronounced changes in motoneuronal conductance due to the oscillation of the membrane potential (see Nelson & Frank 1967; Edgerton et al. 1976). The finding of two EPSPs in the same motoneurone modulated independently of each other eliminate however this possibility (see Fig. 6D). Furthermore the amplitude of conductance pulses (constant current) injected through the microelectrode prior to the stimulus did not change significantly during the cycle (see Fig. 6C). The extracellular field potentials just outside the impaled cells were also routinely

nely recorded (see paper VI)

The phasic modulation of the different EPSPs during fictive locomotion is in accordance with the modulation of the corresponding reflex responses during walking. Since no phasic inflow from the periphery could be present there is clear evidence that the locomotor generator exerts a phasic gating between the different reflex pathways. The most likely explanation would be an interaction on the interneuronal level although there could also be a presynaptic inhibition of alternative sets of peripheral afferents or gating at the dendritic tree of the motoneurons. Two different possibilities may be suggested. First there can be a common last order interneurone on which the efferent signals from the locomotor generator and signals from the reflex pathways converge. This would imply that the signals would mutually facilitate each other before reaching the motoneurons. Such a mechanism can however not explain the modulation of the early extensor EPSP which is largest during the flexor activity. Another possibility would be that the spinal locomotor generator exerts a more selective phasic control with inhibition and/or facilitation of the transmission in specific interneuronal pathways (see Fig. 8). The modulation of the reflex responses is thus at least in part a result of a phasic gating between alternative pathways exerted by the spinal locomotor generator. The excitability level of the motoneurons is inevitably however of major importance for the final outcome of the responses and has thus in itself an effective gating function. The amplitude of the early EPSPs were seldomly modulated more than 40% (see Fig. 7). The low excitability level of the motoneurons reduces certainly the early reflex response of the extensor muscles although the pathway is facilitated during this period by the locomotor generator.

A similar type of phasic gating was demonstrated in the swimming spinal dogfish in which reflex responses were switched between the two sides of the body dependent on the alternating locomotor activity (Grillner et al. 1977; Wallén 1977). Wallén (1979; submitted for publication) showed that undulating movements of the body stabilized the rhythm (see also Grillner & Wallén 1977) but did not influence the reflex responses.

No peripheral feedback was needed for the phasic gating

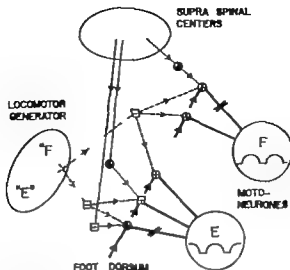


Fig 8 Hypothetical model of the phasic gain control exerted by the spinal locomotor generator and of the upraspinal control on the five reflex pathways from the dorsum of the foot to the flexor and extensor motoneurons. The pathway are drawn as thick line. Plus (+) and minus (-) represent excitatory and inhibitory pathways respectively and the perpendicular lines mark the long latency pathways. The influence from supraspinal centers and from the locomotor generator on the individual pathways plotted as dotted line (plus and minus as above). The influence from the locomotor generator changes in phase with the foot output to the flexor F and extensor E motoneurons.

in the cat (see above). It is however not known whether the phasic inflow during locomotion still assists the centrally exerted gain control or whether it as in the dogfish exerts no or little influence on the actual reflex pathways.

During locomotion in thalassic cats a stimulus of the plantar region of the foot prolonged flexor and extensor activity in the respective phase of the stepcycle (Duyssens & Pearson 1976). A stimulus of the dorsum of the hindfoot evoked during similar conditions an increased flexor or extensor activity in the ipsilateral forefoot depending on the phase of the stepcycle (Miller et al 1977). It was also found that PSPs in flexor and extensor motoneurons evoked from cutaneous sources other than the foot dorsum were phasically modulated during fictive locomotion (Schomburg et al 1977 1978 paper VI). That means that also other reflex pathways are controlled by the locomotor generator.

Phasic gating is not unique for the locomotor system. Brief

chemical stimulation of the carotid body or electrical stimulation of the carotid sinus nerve produced an increased inspiration and an increased discharge in the phrenic nerve during the inspiratory phase of the respiration (Black & Torrance 1967 Band et al 1970 Eldridge 1972a b) but increased the activity of expiratory muscles during expiration (Eldridge 1976) By extracellular recordings from single medullary respiratory neurones which fired during inspiration it was demonstrated that the signals from the chemoreceptors were phasically modulated before projecting to these cells (Lipsky et al 1977) Stimulation of the glossopharyngeal and superior laryngeal nerves evoked short latency excitation of the contralateral phrenic nerve during inspiration but not during expiration (Berger & Mitchell 1976) These responses were also slightly modulated prior to the inspiratory neurones (see above) but the excitability of the motoneurones (phrenic) was suggested to have a more significant function in the phasic gating (Berger 1978)

There is thus in principle the same type of phasic gating in different innate motor behaviours. It is reasonable that gating not is restricted only to these systems but may generally occur concomitant to the efferent output in innate as well as learned central motor programs.

## GENERAL DISCUSSION

The control of movement is one very important function of the central nervous system. The temporal and spatial activation of the motor units that execute a movement is the final result of this control. At the same time as the nervous system controls all motoneurons involved in an ongoing movement, it must transmit and solve a number of other control problems. The more evolutionary advanced an animal, the more complex and the greater the number of mental processes involved. In order for higher levels of the nervous system to solve more intricate problems concomitant with a proper activation of each motor unit, Bernstein (1967) suggested a hierarchical organization of the central nervous system where in the number of parameters controlled by the brain during a movement was reduced. Several autonomic synergisms should be organized at a lower level of the nervous system, ready to execute classes of stereotyped movements when called upon from higher centers in the hierarchy (see also Gelfand et al. 1971).

The present findings are in agreement with such an organization and extend it, since rather complete integrative motor functions can be performed at a low level in the central nervous system (spinal cord). With the specific locomotor activity generated by the spinal cord, a simple and unspecific control from higher levels would suffice to drive the networks faster or slower (see Grillner 1975, 1979b; Shik & Orlovsky 1976). The higher levels would in addition to regulating speed only exert a control of the direction of the locomotion, the adaptation of the movements to the environment and the equilibrium.

## SUMMARY AND CONCLUSIONS

Spinal integrative motor functions involved in the neural control of locomotion have been investigated mainly by analysis of muscular activity and movements in chronic spinal cats

- I In the spinal cord with intact reflex interaction neural networks can generate locomotion that resembles that of intact animals even in many details. The locomotor generators that produce the basic rhythm are influenced from the periphery to provide proper speed adjustments of the locomotor activity. The two limbs are properly coordinated by spinal mechanisms.
- II A basic reflex network that induces tactile placing is organized in the spinal cord. The spinal networks are controlled from supra spinal centers.
- III A stumble corrective reaction is described which is phasically adapted to compensate for unpredicted disturbances of the limb in accordance with the instantaneous and dynamically changing conditions in different phases of the step-cycle. Activity in several spinal reflex pathways contribute to the reaction. The spinal locomotor generator is shown to phasically control individual pathways.



## ACKNOWLEDGEMENTS

It has been a great privilege to have Sten Grillner as a teacher and supervisor. Thank you Sten for your invaluable help and support and for our many inspiring discussions.

I also wish to thank Olle Andersson, Junt Halbertsma, Maria Lindquist, Serge Rossignol and Anders Sjöström for stimulating collaborations and especially Olle and Junt for their introducing me to digital processing technique.

Many people have helped me first in Göteborg and later in Stockholm. Thanks are due to Margreth Svanberg, Maria Wilén and Åke Arvidsson for excellent assistance in experiments and during the preparation of the manuscripts to Erling Eide, Yngve Källström, Harry Hagelin and Georg Görtz for technical construction to Inger Klingebrant and Ulla Siltberg for expert secretarial work to the photographers Tomas Palm and Christian Rosenberg and to the animal keeper Hans Klingebrant.

The valuable help of Michael Zomlefer for scrutinizing the English texts is also gratefully acknowledged.

I also wish to thank Helene for her gracious and unbounded patience, Pelle and Oia for their refreshing diversions and my parents Margit and Lars for their loyal support and for taking care of the boys when part of this summary was written.

The investigation were supported by grants from the Swedish Medical Research Council project nr 14X 3026, The Medical Faculty, University of Göteborg, Karolinska Institutet, Stiftelsen Wilhelm och Martina Lundgrens Vetenskapsfond and Svenska Sällskapet för Medicinsk Forskning.

# REFERENCES

- AMASSIAN V E 1976 The onset of cortical functioning in contact placing by kitten J Physiol (Lond) 263:144-145P
- AMASSIAN V E & ROSS R. 1973 Development in the kitten of control of contact placing by sensorimotor cortex J Physiol (Lond) 230:55-56P
- AMASSIAN V E ROSS R & ZISPEL, B 1977 Dependence of the full contact placing reaction in kitten on supraspinal influences Proc of the International Union of Physiological Sciences XIII:40
- AMASSIAN V E WEINER H & ROSENBLUM M 1972 Neural systems underlying the tactile placing reaction: A model for the study of higher level control of movement B in Res 40 171-178
- ANDERSSON O FORSSBERG H & LINDQUIST M 1977 The neural mechanism of the phase dependent flexor reflex Proc Sixth Sympoium on Neurophysiological Mechanisms of Locomotion Paris
- ANDERSSON O GRILLNER S LINDQUIST M & ZOMLEFER M 1978 Peripheral control of the spinal pattern generators of locomotion in cat Brain Res 150 625-630
- ARSEVSKIY Y I KOTS Y M ORLOVSKIY G M RODIONOV I.M. & SHILK, M.L 1963 Investigation of the biomechanics of running by the dog Biofizika 10 665-672 (English transl 737-746)
- BARD D M CAMERON I R & SEMPLE S J G 1970 The effect on respiration of abrupt changes in tidal volume pH and  $P_{CO_2}$  in the cat J Physiol (Lond) 211 479-494
- BARD P 1933 Studies on the cerebral cortex 1 Localized control of placing and hopping reactions in the cat and their normal management by small cortical remnant Arch Neurol Psychiat 30 40-74
- BARD P 1938 Studies on the cortical representation of somatic sensibility "The Harvey Lectures The Williams and Wilkins Company Baltimore 33 143-169

- BAIRD P BROOKS C M and LOWRY T 1932 Cerebral localization of "hopping and placing reactions in cats and lizards Amer J Physiol 101:3-4
- BENTLEY B & KONISHI M 1978 Neural control of behaviour Ann Rev Neurosci 1:35-60
- BERGER, A J 1978 Respiratory gating of phrenic motoneuron responses to superior laryngeal nerve stimulation B in Res 157:381-384
- BERGER A J & MITCHELL E A 1976 Later fixed phrenic nerve responses to stimulating respiratory afferents in the cat Am J Physiol 230:1314-1320
- BERNSTEIN N 1967 The coordination and regulation of movements H. K. Simon Press Oxford
- BIGHALL K E 1974 Ontogeny of levels of neural organization: The righting reflex as a model Exp Neurol 42:566-573
- BLACK A M S & TORRANCE R W 1967 Chemoreceptor effects in the respiratory cycle J Physiol (Lond) 189:59-61P
- BOGEN J E & CAMPBELL B 1962 Recovery of foreleg placing after ipsilateral frontal lobectomy in the hemi-parietal cat Science 135:309-310
- BRADLEY G W EULER C V MARTILA I & ROOS B 1975 A model of the entrained and reflex inhibition of inspiration in the cat Biol Cybernetics 19:103-116
- BROOKS C M 1933 Studies on the cerebral cortex II Localized representation of hopping and placing reactions in the rat Amer J Physiol 105:162-171
- BROOKS C & WOOLSEY C M 1936 Relations in the rabbit of electrically excitable areas for placing and hopping reactions Amer J Physiol 116:17-18
- BROWN T G 1911 The intrinsic factors in the state of progression in the mammal Proc Roy Soc Lond B 84:308-319
- BROWN T G 1914 On the nature of the fundamental activity of the nervous centre together with an analysis of the conditioning of rhythmic activity in progression and a theory of the evolution of function in the nervous system J Physiol (Lond) 48:18-46
- BUDAKOVA, N N 1973 Stopping movements in the spinal cat due to DOPA administration Fiziol Zh USSR 59:1190-1198

- BURKE R.E. JANKOWSKA, E. & ten BRUGGEMATE C 1970 A comparison of peripheral and rubrospinal synaptic input to slow and fast twitch motor units of triceps surae J Physiol (Lond) 207 709-732
- BURKE R.E. MYER W.E. & WALSH J.V.-Jr 1973 Functional specialization in the motor unit population of cat medial gastrocnemius muscle. In Control of Posture and Locomotion Eds R.B. Stein, K.G. Pearson, R.S. Smith and J.B. Redford Plenum Press New York 29-44
- CHAMBERS W.W. 1953 Role of cerebellum in placing reactions of the Feline. Fedn Proc Fedn Am Soc exp Biol 12:25
- CHAMBERS W.W. & SPRAGUE J.M. 1955 Functional localization in the cerebellum - I. Organization in longitudinal corticonuclear zones and their contribution to the control of posture both extra-pyramidal and pyramidal J Comp Neurol 103 105-129
- COHEN A.H. 1979 Relationship between forelimb coordination and movement asymmetries during fast gaits canter and gallop Brain Res 164:332-356
- COOPER S. DENNY-BROWN D.E. & SHERRINGTON C.S. 1927 Interaction between ipsilateral spinal reflexes acting on the flexor muscles of the hindlimb Proc R Soc Lond B 101:262-303
- CONVAJA M. GROFOVA, I. POMPEIANO O. & WALBERG F. 1977 The lateral reticular nucleus in the cat - II Effects of lateral reticular lesions on posture and reflex movements Neuroscience 2:929-943
- CREED R.S. DENNY-BROWN D.E. ECCLES J.C. LIDDELL E.C.T. & SHERRINGTON C.S. 1932 Reflex activity of the spinal cord Oxford Univ Press London
- DELLON P.G. & LUND J.P. 1971 Evidence for entrained timing of rhythmic oscillation J Physiol (Lond) 213:1-13
- DENNY-BROWN D. & CHAMBERS R.A. 1958 The parietal lobe and behaviour Res Publ Ass Nerv ment Dis 36:35-117
- DOMATELLE J.M. 1978 Growth of the corticospinal tract and the development of placing reactions in the postnatal rat J Comp Neurol 175 207-232
- DUTSIS J. & PEARSON K.G. 1976 The role of cutaneous afferents from the distal hindlimb in the regulation of the step cycle in thalamic cats Exp Brain Res 24:245-255

- ECCELES R & LUNDBERG A 1959 a Synaptic actions in motoneurons by afferents which may evoke the flexion reflex Arch Ital Biol 97:199-221
- ECCELES R & LUNDBERG A 1959 b Supraspinal control of interneurons mediating spinal reflexes J Physiol (Lond) 147:565-584
- EDGERTON V R GRILLNER S SJÖSTRÖM A & ZANGGER H 1976 Central generation of locomotion in vertebrates. In "Neural Control of Locomotion" Eds R. Herman, S. Grillner, P. Stein and D. G. Stuart. Plenum Press, New York, 439-464
- EIAL-EINSENFELDT I 1975 Ethology: The biology of behavior. Holt, Rinehart and Winston, Cop. New York
- EICHHORST H & MAUNYH B 1874 Ueber die Regeneration und Veränderungen im Rückenmark nach Stückenwei totaler Zerstörung desselben. Maunyn-Schmiedeberg Arch f exp Pathol und Pharmak 2:225-253
- ELDRIDGE F L 1972 a The importance of timing on the respiratory effects of intermittent carotid sinus nerve stimulation J Physiol (Lond) 222:297-318
- ELDRIDGE F L 1972 b The importance of timing on the respiratory effects of intermittent carotid body chemoreceptor stimulation J Physiol (Lond) 222:319-333
- ELDRIDGE F L 1976 Respiratory effects of brief carotid sinus nerve and carotid body stimulation Respi Physiol 26:395-410
- ENGBERG I 1964 Reflexes to foot muscles in the cat Acta Physiol Scand 62:suppl 235
- ENGBERG I & LUNDBERG A 1969 An electromyographic analysis of muscular activity in the hindlimb of the cat during unrestrained locomotion Acta Physiol Scand 75:614-630
- ENGBERG I, LUNDBERG A & KYALL R W 1968 Reticulospinal inhibition of transmission in reflex pathways J Physiol (Lond) 194:201-223
- ENGLISH A W 1979 Interlimb coordination during stepping in the cat: an electromyographic analysis J Neurophysiol 42:229-243
- FORSBERG B & GRILLNER S 1973 The locomotion of the cat spinal cat injected with Clonidine i.v. Br J Pharmacol 50:184-186

- FORSSBERG H GRILLNER S & ROSSIGNOL S 1973 Phase dependent reflex reversal during walking in chronic spinal cats. *Brain Res* 85:103-107
- FREUSBERG A 1974 Reflexbewegungen beim Hund. *Pflüg Arch ges Physiol* 9:358-391
- GAMBARIAN P P ORLOVSKY M M PROTOPOPOVA T Y SKVERIN V V & SHIK, M. I. 1971 The activity of muscles during different gaits and adaptive changes of moving organs in family Felidae. Morphology and ecology of vertebrates. *Proc Inst Zool Acad Sci USSR* 48:220-239
- GELFAND I M GURFINKEL V S TSETLIN M L & SHIK M. I. 1971 Some problems in the analysis of movements. In "Models of the structural-functional organization of certain biological systems". Eds I. M. Gelfand, V. S. Gurfinkel, S. V. Fomin and M. L. Tsetlin. MIT Press, Cambridge, Mass. 329-345 (Translated from Russian, original publ. 1966)
- GELFAND I M & TSETLIN M L 1971 Mathematical modeling of mechanisms of the central nervous system in certain biological systems. In "Models of the structural-functional organization of certain biological systems". Ed. I. M. Gelfand, V. S. Gurfinkel, S. V. Fomin and M. L. Tsetlin. MIT Press, Cambridge, Mass. 1-22 (Translated from Russian, original publ. 1966)
- GILMAN S & MARCO L A 1971 Effects of medullary pyramidotomy in the monkey. I. Clinical and electromyographic abnormalities. *Brain* 94:495-514
- GOSLOW G E J REINKING R M & STUART D G 1973 The cat step cycle. Hindlimb joint angles and muscle lengths during unrestrained locomotion. *J Morphol* 141:1-42
- GOTTLIKE G L & AGARHALL G C 1970 Filtering of electromyographic signal. *Amer J Physiol Med* 49:142-146
- GRILLNER S 1969 Supraspinal and segmental control of static and dynamic  $\alpha$ -motoneurons in the cat. *Acta Physiol Scand* suppl. 327
- GRILLNER S 1972 The role of muscle stiffness in meeting the changing postural and locomotor requirements of the developing animal. *Acta Physiol Scand* 86:92-108
- GRILLNER S 1973 Locomotion in the spinal cat. I. "Control of Posture and Locomotion". Ed. R. B. Stein. In K. G. Paarsen, R. S. Smith and J. H. Redford. Plenum Press, New York. 515-535

- GRILLNER S 1975 Locomotion in vertebrates: Central mechanisms and reflex interaction Physiol Rev 55:247-304
- GRILLNER S 1979 a Interaction between central and peripheral mechanisms in the control of locomotion Progr Brain Res (in press)
- GRILLNER S 1979 b Control of locomotion in bipedal tetrapods and fish In "Handbook of Physiology" Ed V Brooks III (in press)
- GRILLNER S, FERRET C & ZANGGER P 1976 Central generation of locomotion in the spinal dogfish Brain Res 109:255-269
- GRILLNER S & ROSSIGNOL S 1978 On the initiation of the swing phase of locomotion in chronic spinal cats Brain Res 146:269-277
- GRILLNER, S, ROSSIGNOL S & WALLÉN P 1977 The adaptation of a reflex to the ongoing phase of locomotion in fish Exp Brain Res 30:1-11
- GRILLNER S & SHIK, M L 1973 On the descending control of the lumbo sacral spinal cord from the "mesencephalic locomotor region" Acta Physiol Scand 87:320-333
- GRILLNER S & WALLÉN P 1977 Is there a peripheral control of the central pattern generators for swimming in dogfish? Br J Res 127:291-295
- GRILLNER, S & ZANGGER P 1974 Locomotor movements generated by the deafferented spinal cord Acta Physiol Scand 91:38-39A
- GRILLNER S & ZANGGER P 1975 How d-tailed is the entrained pattern generation for locomotion? Brain Res 88:367-371
- GRILLNER S & ZANGGER P 1979 On the central generation of locomotion in the low spinal cat Exp Brain Res 34:241-261
- HAGBARTH K E 1952 Excitatory and inhibitory skin areas for flexor and extensor motoneurons Acta Physiol Scand 26: suppl 34
- HALBERTEMA J.M 1976 Locomotion of the cat stepping on a treadmill: timing in limb flexion and extension Delft University of Technology Dept of Applied Physics Prosthetics Control Laboratory Internal Report

- HALBERTSMA J.M. MILLER S & VAN DER MECHÉ F O.A 1976 Basic programs for the phasing of flexion and extension movements of the limbs during locomotion In "Neural Control of Locomotion" Eds R Hartman, S Grillner, P Stein and D Stuart Plenum Press New York 489-518
- HARLOW H F & HARLOW M K 1962 Principles of primate learning In "Little Club Clinics in Developmental Medicine" Hainemann London No 7
- HEKHTMAN E 1974 Motor functions of the brain stem and basal ganglia In "Medical Physiology" Ed V B Mountcastle Mosby Company St Louis P 695
- HICKS S P & D'AMATO C.J 1975 Motor-sensory cortex-corticospinal systems and developing locomotion and placing in rats *Am J Anat* 143:1-42
- HINDE R A 1966 Animal behaviour: A synthesis of ethology and comparative psychology McGraw-Hill Book Company London
- HOLMQVIST B & LUNDBERG A 1961 Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alpha motoneurons *Acta Physiol Scand* 54: suppl 186
- HOLST von E 1939 Die relative Koordination. *Ergbn Physiol* 42:228-306
- HONGO T JANKOWSKA E & LUNDBERG A 1969 The rubrospinal tract II Facilitation of interneuronal transmission in reflex paths to motoneurons *Exp Brain Res* 7:365-391
- JANKOWSKA E JUKES M.G.M LUND S & LUNDBERG A 1967 The effect of DOPA on the spinal cord 5 Reciprocal organization of pathways transmitting excitatory action to alpha motoneurons of flexor and extensors *Acta Physiol Scand* 70 369-388
- JANKOWSKA E JUKES M.G.M LUND S & LUNDBERG A 1967 b The effect of DOPA on the spinal cord 6 Half-centre organization of interneurons transmitting effects from the flexor reflex afferent *Acta Physiol Scand* 70:398-402
- KELLOGG W M DEESE J & PROKTO M N 1946 On the behavior of the lumbo-spinal dog *J Exp Psychol* 36:503-511
- KILGIN A S & SHIK M L 1970 Interaction of symmetrical limbs during controlled locomotion *Biolika* 15:164-170 (Engl transl 171-178)



- LIPSKI J McALLEN R M & SPYER, K M 1977 The carotid chemoreceptor input to the respiratory neurones of the nucleus of tractus solitarius *J Physiol (Lond)* 269:797-810
- LUMLEY J S P 1973 The role of the postcentral gyrus in the tactile crossed placing reaction in the Rhesus monkey *Brain* 96:827-832
- LUNDBERG A 1973 The significance of segmental spinal mechanisms in motor control *Proc Symp Papers 4th Int Biophysic Congr Moscow*
- LUNDBERG A & WORSSELL U 1960 Spinal afferent pathway of the tactile placing reaction *Experientia* 16:123-124
- LUNDBERG A & VOORHIEVE P 1962 Effects from the pyramidal tract on spinal reflex arcs *Acta Physiol Scand* 36:201-219
- MACHWIS R 1924 *Körperstellung* Verlag von Julius Springer Berlin
- MALING R.M & ACHESON G H 1946 Righting and other postural activity in low decerebrate and in spinal cat after d-amphetamine *J Neurophysiol* 9:379-386
- MANTER J T 1938 The dynamics of quadrupedal walking *J Exp Biol* 15:522-540
- MARZONI D POMPEIANO O & STAMPACCHIA G 1979 Cervical control of posture and movement *Brain Res* 169:613-619
- MARREY E J 1895 *Movement* London
- MASSION J & SMITH A M 1973 Activity of ventrolateral thalamic neurons related to posture and movement during contact placing response in the cat *Brain Res* 61:400-406
- MASSION J & SMITH A M 1974 Ventrolateral thalamic neurons related to posture during a modified placing reaction *Brain Res* 71:353-359
- MATTHEWS B M. 1972 Simple networks *Ann N Y Acad Sci* 193:59-72
- MEYER F.M BOWEL J.A & MEYER D R 1963 Effects of d-amphetamine upon placing responses in neocortical cat *J Comp Physiol Psychol* 56:402-404
- MILLER A J 1972 Significance of sensory inflow to the swing limb *Brain Res* 43:147-159
- MILLER S RUIT J B & VAN DER MEERE F G.A 1977 Reversal of sign of long spinal reflexes dependent on the phase of the step-cycle in the high decerebrate cat *Brain Res* 128 447-459

- MILLER S & VAN DER MECHT F G A 1976 Coordinated stepping of all four limbs in the high spinal cat. *Brain Res* 109:395-398
- NELSON P G & FRANK, K 1967 Anomalous rectification in cat spinal motoneurons and effect of polarizing currents on excitatory postsynaptic potential. *J Neurophysiol* 30:1097-1113
- ORLOVSKY G W 1970 Activity of reticulo-spinal neurons during locomotion. *Biofizika* 15:728-737 (Engl transl 761-771)
- ORLOVSKY G W 1972 Activity of rubro-spinal neurons during locomotion. *Brain Res* 46:99-112
- PEARSON K G 1972 Central programming and reflex control of walking in the cockroach. *J Exp Biol* 56:173-193
- PEARSON K G & DUTSERS J 1976 Function of segmental reflexes in the control of stepping in cockroaches and cats. In "Neural Control of Locomotion" Eds R Herman, S Grillner. P. Stein and H Stuart. Plenum Press New York 18:519-538
- PERRET C & CABELGUEN J M 1975 A new classification of flexor and extensor muscles revealed by study of the central locomotor program in the deafferented cat. *Exp Brain Res suppl* 160
- PHILIPPOU M 1905 L'antonomie de l'entraineement dans le système nerveux des animaux. *Trav Lab Physiol Inst Solvay Bruxelles* 11 7:1-208
- POMPEIANO O 1967 Functional organization of the cerebellar projections to the spinal cord. In *Progress in Brain Research* Eds C.A. Fox and R.S. Snid. Elsevier Amsterdam 282-321
- PROCHAZKA A, SONTAG K.-H & WAND P 1978 Motor reactions to perturbations of gait: proprioceptive and somesthetic involvement. *Neurosci Lett* 7:35-39
- RADEMAKER G C J 1931 *Das Stehen*. Verlag von Julius Springer Berlin
- RADEMAKER, G C J & HOOGERWERF S 1930 Réactions provoquées par l'allongement passif d'un muscle, semi-tendineux. *Arch néerl de physiol* 15:338-357
- RASMUSSEN S, CHAN A K & COSLOW G R Jr 1978 The cat step cycle: Electromyographic patterns of hindlimb muscles during posture and unrestrained locomotion. *J Morph* 155:253-270
- ROBERTS T.D.M 1978 *Neurophysiology of postural mechanisms*. Battisworth London

- ROSSIGNOL S GRILLNER S & FORSSBERG H 1975 Factors of importance for the initiation of flexion during walking Neurosci Abstr 1:181
- SCHOMBURG E D & Behrends H B 1978 Phase-dependent transmission in the segmental pathways from cutaneous afferents to  $\alpha$ -motoneurons during fictive locomotion Pflügers Arch 373: suppl R 70
- SCHOMBURG E D ROSELER J & MEINCK H-M 1977 Phase-dependent transmission in the excitatory propriospinal reflex pathway from forelimb afferents to lumbar motoneurons during fictive locomotion Neurosci Lett 4:249-252
- SILVERSTEIN A I 1976 Neuronal mechanisms for rhythmic motor pattern generation in a simple system In "Neural Control of Locomotion" Eds E. Herrman, S. Grillner, P. Stein and D. Stuart Plenum Press New York 18:377-399
- SHERINGTON C S 1910 Flexion-reflex of the limb, crossed extension-reflex and reflex stepping and standing J Physiol (Lond) 40:128-121
- SHERINGTON C S 1947 The integrative action of the nervous system Yale University Press New Haven
- SHERINGTON C S & SOWTON J C M 1911 a Chl. reflex and reversal of reflex effect J Physiol (Lond) 42:383-388
- SHERINGTON C S & SOWTON J C M 1911 b Reversal of the reflex effects of an afferent nerve by altering the character of the electrical stimulus applied Proc Roy Soc London B 83:435-446
- SHIK M L & OKLOVSKY G M 1965 Biophysics of complex systems and mathematical models Co-ordination of the limb during running of the dog Biofizika 10:1037-1047 (engl. transl. 1148-1159)
- SHIK, M L & OKLOVSKY G M 1976 Neurophysiology of locomotor automation Physiol Rev 55:465-501
- SHIMAMURA M & LIVINGSTON R B 1963 Longitudinal conduction systems serving spinal and brain-stem coordination J Neurophysiol 26:258-272
- SHREVEGER P S & DYKMAN R A 1951 Walking: spinal carnivores J Comp Physiol Psychol 44:252-262

- SMITH J.L. EDGERTON V.R. BETTS B. & COLLATOS T.C. 1977  
EMG of slow and fast ankle extensors of cat during posture locomotion and jumping J Neurophysiol 40:503-513
- SWIDER R.S. 1940 Functional studies following lesions of the nucleus interpositus in the rabbit Johns Hopkins Hosp Bull 67:139-153
- STEIN P.S.G. 1976 Mechanisms of interlimb phase control In "Neural Control of Locomotion" Eds R. Herman & Grillner P. Stein and D. Stuart Plenum Press New York 18 463-487
- STEINER D.J. FESHLER, W.B. & WEBER E.D. 1975 Effects of spinal transection in neonatal and weanling rats: Survival and function Exp Neurol 46:156-177
- STUART D.G. WITNEY T.P. WHITEL H.L. & COSLOW B.E.J. 1973 Time constraints for inter-limb co-ordination in the unrestrained locomotion. In "Control of Posture and Locomotion" Ed. R.B. Stein, K.G. Pearson R.S. Smith and J.B. Redford Plenum Press New York 537-560
- STOKELY G. CZER G. & VÖRÖS B. 1969 Activity pattern of limb muscles in freely moving normal and deaf-mutated newts Exp Brain Res 9 53-62
- TANG Y. 1935 On the development of different placing reactions in the albino rat Chin J Physiol 9:339-346
- ten CATE J. 1940 Quelques observations sur la locomotion des chiens dont la moelle épinière est sectionnée transversalement Arch Neerl Physiol 24 476-485
- ten CATE J. 1962 Innervation of locomotor movements by the lumbosacral cord in birds and mammals J Exp Biol 39 239-242
- ten CATE J. 1964 Locomotory movements of the hind limb in rabbits after isolation of the lumbosacral cord J Exp Biol 41 359-362
- THORPE W.H. 1964 Learning and instinct in animal Methuen London
- TIMBERGEN W. 1951 The study of instinct Clarendon Press Oxford
- TOKURIKI M. 1973 Electromyographic and joint-mechanical studies of quadrupedal locomotion J Walk Jap J vet Sci 33 433-446

- TOWER S S 1940 Pyramidal lesion in the monkey Brain 63:36-90
- WALLEN H 1977 On the mechanism of the phase dependent reflex reversal in the swimming fish Proc XVIIth International Congress of Physiological Sciences Paris XIII 2377
- WALMSLEY B HODGSON J A & BURKE R E 1978 Forces produced by medial gastrocnemius and soleus muscles during locomotion in freely moving rats J Neurophysiol 41:1203-1216
- WEBER, E D & STELZNER D J 1977 Behavioral effects of spinal cord transection in the developing rat Brain Res 125:241-255
- VENTINK, G H 1976 The action of the hind limb musculature of the dog in walking Acta nat 96:70-80
- WETZEL M C & STUART D G 1976 Ensemble characteristic of cat locomotion and its neural control In Progr Neurobiol Pergamon Press New York 7:1-98
- VIALA H & BUSER P 1969 The effects of DOPA and 5-HTP on rhythmic afferent discharges in hindlimb nerves in the rabbit Brain Res 12:437-443
- VIDAL C VIALA D & BUSER P 1979 Central locomotor programming in the rabbit Brain Res 168:57-73
- WIERSMA C A G & IKEDA K 1964 Interneurons commanding swimmeret movements in the crayfish Procamarus Clarkii (Girard) Comp Biochem Physiol 12:309-325
- WILSON D M 1961 The central nervous control of flight in a locust J Exp Biol 38:471-490
- WILSON E O 1975 Sociobiology The new synthesis The Belknap Press of Harvard University Press Cambridge Mass
- WOOLSLEY C W & BAIRD P 1936 Cortical control of placing and hopping reactions in Macaca mulatta Ann J Physiol 116:163-166





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 475

Variation of Serum Gastrin Concentration  
Trophic Effects on  
the Gastrointestinal Tract  
of the Rat

J. OSCARSON, R. HÅKANSON, O. LIEBERG,  
O. LUNDQVIST, F. SUNDLER and J. THORELL

LUND 1979





From the Departments of Surgery, Pharmacology and  
Histology University of Lund Lund and the  
Department of Clinical Chemistry Academic Hospital,  
University of Uppsala, Uppsala and the Department  
of Nuclear Medicine University of Lund at Malmö  
General Hospital Malmö, Sweden

# Variated Serum Gastrin Concentration Trophic Effects on the Gastrointestinal Tract of the Rat

BY

J. OSCARSON, R. HÅKANSON, G. LIEBERG,  
G. LUNDQVIST, F. SUNDLER and J. THORELL

LUND 1979

Distributor: Almqvist & Wiksell Tryckeri AB, Rapsgatan 8, S-751 81 Uppsala, Sweden

From the Departments of Surgery Pharmacology and  
Histology University of Lund Lund and the  
Department of Clinical Chemistry Academic Hospital,  
University of Uppsala, Uppsala and the Department  
of Nuclear Medicine University of Lund at Malmö  
General Hospital Malmö Sweden

Abstract

OSCARSON J., HÅKANSON H. LIEBERG E. LUNDQVIST G.  
SUNDLER F. and THORELL J. *Varied serum gastrin concentration.  
Trophic effects on the gastrointestinal tract of the rat*  
*Acta physiol scand 1979 Suppl 475*

Gastrin is thought to be a trophic agent for the digestive tract and the pancreas. This concept was studied on rats subjected to various operations designed to create hyper- or hypo-gastrinemia. Elevated serum gastrin concentration resulted from vagal denervation, antrum exclusion and fundectomy. Reduced serum gastrin concentration resulted from antrectomy. Antrum exclusion but not vagotomy increased the weight and height of the oxyntic mucosa; antrectomy had the opposite effects. There were no trophic changes in the small and large bowel or pancreas attributable to the serum gastrin concentration. In the bypassed duodenum (following antrum exclusion or antrectomy II II) the villus height was greatly lowered suggesting that in this location the passage of food acts as a trophic stimulant. Our results show that endogenous gastrin exerts a trophic effect on the oxyntic mucosa; there was no evidence for a similar effect on the extragastric gut and pancreas.

**Key-words** gastric surgery hypergastrinemia hypogastrinemia  
trophic action digestive tract

## INTRODUCTION

Gastrin is a physiological stimulant of gastric HCl. Among other actions of exogenous gastrin and its analogues are trophic effects on the mucosa of the digestive tract except for the esophagus and gastric antrum (Johnson et al 1975 Barrowman 1975 Johnson 1976 Mak and Chang 1976). A trophic effect of gastrin on the pancreas has also been advocated (Barrowman 1975; Johnson 1976 Mayston and Barrowman 1973 Mayston et al 1975 Reber et al 1977). It has been argued that the trophic actions of gastrin are to be regarded as physiological (Johnson 1976 1977). Other gastrointestinal hormones have also been claimed to affect tissue growth and cell renewal (Johnson and Guthrie 1976). Exogenous cholecystokinin produces hypertrophy and hyperplasia of the pancreas (Petersen et al 1978). Secretin (Johnson and Grossman 1969) and somatostatin (Bloom et al 1974 Gomez-Pan et al 1975 Barros D Sa et al 1975, Arnold et al 1975 Konturek et al 1976 Vatn et al 1977 Phillip et al 1977) inhibit the secretagogue effects of gastrin. Secretin (Stanley et al 1972 Wiseman and Johnson 1976) and possibly somatostatin as well, counteracts the trophic action of gastrin. It is not yet clear which of these trophic or antitrophic effects should be considered physiological; so far endogenous gastrin has not been shown to have all the effects ascribed to exogenous gastrin. Thus in patients with gastrinoma hyperplasia was observed in oxyntic (Neuburger et al 1972) and duodenal mucosa but none was noted in ileal mucosa (Shimoda et al 1968 Mansbach et al 1968). Variations in the serum gastrin concentration affected neither the starvation atrophy of the small bowel nor the hyperplastic response after jejunoctomy (Oscarson et al 1977). No effect was noted in small and large bowel mucosa after longstanding changes in the serum gastrin concentration (Oscarson et al 1979).

Treatments that raise or lower the serum gastrin concentra-

tion have been found to affect the oxyntic gland area of the stomach in various ways. A correlation between endogenous serum gastrin levels and rate of synthesis of DNA has been reported (Willems et al 1977, Casteleyn et al 1977). Upon transposition or exclusion of the antrum the height of the oxyntic mucosa was increased as was also the number of parietal cells, chief cells (Lehy et al 1973) and endocrine cells (Alumets et al 1979). Antrectomy which results in hypogastrinemia reduced the size (Martin et al 1970) and the height of the oxyntic mucosa (Lehy et al 1973), the size and volume density of parietal cells (Capoferro and Nygaard 1973, Helander 1976) and the number of endocrine cells (Håkanson et al 1976).

Available information suggests that endogenous gastrin exerts trophic control of the oxyntic mucosa, possibly also of the duodenum, while its importance for the small and large bowel and for the pancreas is doubtful. In the present communication we describe the effects on gut and pancreas of various gastric operations resulting in widely differing serum gastrin concentrations.

#### MATERIAL AND METHODS

Male Sprague-Dawley rats weighing 200-250 g purchased from the same breeder were used. The rats were housed in plastic cages and given commercial rat food pellets and water. The animal quarters were lighted in alternate cycles. The rats were randomly allocated for the experimental groups. Control rats were unoperated. Vagotomy was abdominal and the added pyloroplasty was in the Heinecke-Mikulicz fashion. One group of rats had pyloroplasty only. Antrectomy was carried out by removal of the distal half of the glandular stomach including the entire lesser curvature followed either by gastroduodenostomy end-to-end (Billroth I) or gastrojejunostomy end-to-side (Billroth II). Antrum exclusion was performed by transecting the stomach between the oxyntic and antral mucosa.

closing the antrum and reestablishing the gastrointestinal continuity by a gastrojejunostomy end-to-side. The vagal innervation was spared. Fundectomy involved resection of the entire oxyntic gland area, followed by plication of the rumen and anastomosis of the antrum to it; vascular and vagal trunks were preserved (Fig. 1).

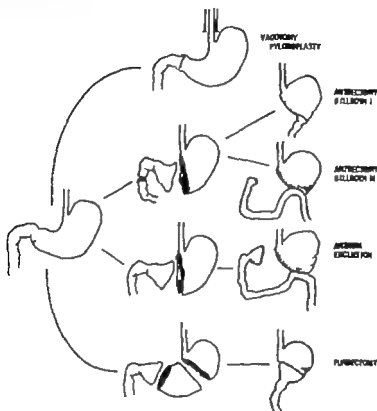


Fig. 1. Schematic presentation of vagal denervation and the various gastric resections.

Food was withdrawn the evening before operation and feeding was resumed the following day. Operations were performed under diethyl ether anesthesia. No antibiotics were used. Survival rate varied from 53 to 100% (Appendix Table I). Postoperative weight gain was similar in all groups with the exception of vagotomy (see also Mordes et al. 1977) (Appendix: Fig. 1). Ten weeks after the operation half the number

of rats in each group were fasted for 48 hours in individual cages with wire mesh bottoms the others were freely fed. The animals were killed between 10 a.m. and 12 noon by exsanguination from the abdominal aorta under light diethyl ether anesthesia. Serum and plasma (taken in heparinized tubes containing 400 KIE Trasylol<sup>R</sup> per ml blood) was stored at  $-30^{\circ}\text{C}$ . The pancreas was dissected out in toto frozen on dry ice and weighed. The stomach was removed, opened along the major curvature, rinsed with iced 0.9% NaCl solution and weighed. The opened stomach was flattened on a wooden board with the mucosal surface upwards and a standard punch (5 mm in diameter) was taken with a punch press from a predetermined site on the oxyntic gland area (Fig. 2).

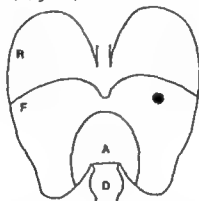


Fig. 2 Schematic presentation of the site and size of the fundic punch. R: rumen; F: fundus (oxyntic gland area); A: antrum; D: duodenum.

The punch was weighed and frozen in liquid propane-propylene for histological examination (see below). The oxyntic and antral mucosa were scraped separately off the gastric wall with a scalpel, frozen on dry ice and weighed. From the duodenum two specimens (each one cm long) were taken: one immediately below the duodenal bulb (proximal duodenum) and one 5 cm more distally (distal duodenum). They were rinsed in iced 0.9% NaCl solution, gently blotted, weighed and frozen for histology in liquid propane-propylene.

The small and large bowel were removed and specimens (5 cm

in length) were excised from the mid small and mid large bowel gently flushed with iced 0.9% NaCl solution opened and the mucosa was scraped off the muscular wall with glass slides. The mucosal scrapings were frozen on dry ice and weighed. Material for chemical analysis was stored at  $-30^{\circ}\text{C}$ .

Mucosal scrapings and pancreas were thawed and homogenized in 5% (w/v) citric acid in 0.25 M sucrose. RNA and DNA were measured by the technique of Scott et al. (1956) as modified by Hinrichs et al. (1964). Aliquots of the homogenates were extracted with cold 0.3 N perchloric acid (PCA). The combined supernatants were washed with cold 80% ethanol and with a mixture of equal parts of ethanol and diethyl ether; the supernatants obtained contained mostly lipids. The residues were then digested in 1 N NaOH for 1 hour at room temperature. Upon addition of 6 N HCl, DNA and proteins precipitated leaving the RNA fraction in the supernatant. The residues were washed with a 1:5 mixture of 6 N HCl and 1 N NaOH and this wash was added to the RNA fraction. The residues were digested in 1 N PCA at  $60^{\circ}\text{C}$ . The digestion was repeated and the supernatants combined to constitute the DNA fraction. The amount of RNA and DNA in tissue were determined by ultraviolet spectrophotometry.

Protein in the crude tissue extracts was measured with the colorimetric method of Lowry et al. (1951).

For histological examination the specimens frozen in propane-propylene were freeze-dried, fixed in formaldehyde vapor (Björklund et al. 1972) and embedded in paraffin. Sections were cut perpendicular to the mucosal surface and stained with hematoxylin and eosin. Examination was with objective  $\times 63$  and eyepiece  $\times 8$  (visual field diameter 2.5 mm). At least 10 determinations of oxyntic mucosal height and proximal and distal duodenal villus height and crypt depth were made on at least 2 sections from each animal.

Gastrin in serum was measured using the radioimmunoassay described by Stadil and Rehfeld (1971, 1973). Antibodies



against synthetic human gastrin I (residues 2-17) were supplied by Professor Jens Rehfeld Institute of Medical Biochemistry University of Aarhus Denmark Radiiodinated heptadecapeptide gastrin I was prepared by the technique of Stadil and Rehfeld (1972) Incubation was at 4°C for at least 48 hours Free tracer was separated from bound by the addition of anion exchange resin as described by Rehfeld and Stadil (1973) Results were expressed as pg equiv synthetic human gastrin I per ml

Plasma somatostatin was determined by a solid phase radioimmunoassay (Arimura et al 1979) with somatostatin antibodies (a generous gift from Dr Robert Elde Department of Anatomy University of Minnesota Minneapolis USA) coupled to cyanogen bromide activated cellulose according to Wide (1969) The antiserum has been characterized earlier with respect to its crossreactivity against different parts of the somatostatin molecule (Arimura et al 1979) as well as to its lack of crossreactivity against other gastrointestinal peptides (Lundqvist et al 1979) Before radioimmunoassay plasma samples were extracted with acetone-petroleum ether as previously described (Arimura et al 1979) Results were expressed as pg equiv synthetic ovine somatostatin per ml

Statistical significance was assessed by analysis of variance Rats with no operation were used as control If heterogeneity of group means between treatment group was observed the confidence interval at  $p = 0.99$  for the control group was calculated Means outside this confidence interval were considered different from the control Vagotomy and pyloroplasty was compared to pyloroplasty alone

## RESULTS

Gastrin and somatostatin in blood The diurnal variation in serum gastrin concentration of unoperated freely fed rats was followed in one experiment which showed about 3 times difference between zenith and nadir; zenith was at about 12

midnight and nadir at about 8 a m (Fig 3)

In the operated rats the highest serum gastrin concentration was observed after fundectomy (3.5 times higher than in freely fed unoperated animals) (Fig 4) while the lowest serum gastrin concentrations were recorded after antrectomy (10-20 per cent of that in freely fed unoperated animals). In the freely fed state the serum gastrin concentration was about the same in unoperated rats and following pyloroplasty, vagotomy and antrum exclusion. In unoperated rats fasting for 48 hours lowered the serum gastrin level to about 20% of that in the freely fed state. Fasting did not lower the serum gastrin level after antrum exclusion or fundectomy. After vagotomy fasting induced only a slight reduction ( $p < 0.02$ ).

Although the plasma somatostatin concentrations generally tended to be higher in fed than in fasted animals (Appendix: Table II) there was no correlation with the serum gastrin concentration. There was a tendency for the various gastric resections to lower the plasma somatostatin concentration (in freely fed rats).

**Organ weight and mucosal height.** The stomach was heavier after vagotomy than after pyloroplasty alone (Fig 5). This increase was not in the weight and height of the oxyntic mucosa (Fig 6). Also the antral mucosa was slightly heavier after vagotomy than after pyloroplasty alone (Fig 5). After antrum exclusion a pronounced increase was noted in the weight of the fundic punch (Fig 5). The oxyntic mucosa was higher in antrum excluded rats than in unoperated rats and lower after antrectomy (Fig 6).

Specimens from the bypassed duodenum (after antrectomy BII or after antrum exclusion) weighed less than specimens from the duodenum left in continuity (after antrectomy BI) (Fig 7). No other surgical procedure significantly affected the weight of the duodenal wall. Also the villus height of the duodenal mucosa was greatly reduced after antrum exclusion and antrectomy BII while after antrectomy BI no decrease

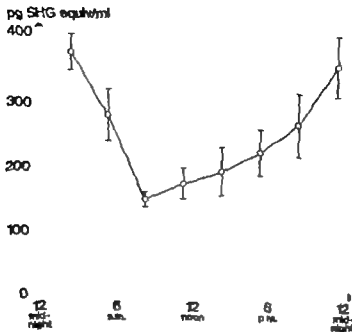


Fig 3 Diurnal variation of serum gastrin concentration of freely fed rats  $n=5$  for each time

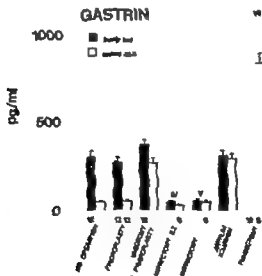


Fig 4 The serum gastrin concentration of the various experimental groups  $p < 0.01$  for III v II (freely fed)  $< 0.001$  v II (fasted)  $p < 0.001$  for IV and V v I (freely fed)  $p < 0.001$  for VI v I (fasted)  $p < 0.001$  for VII v I



Fig 5 Weight of the whole stomach (a) oxyntic (b) and antral (c) mucosa and of the fundic pouch (d) Antrum excision and antrectomy are not included because of the error introduced by the resection  $p < 0.001$  for IIIa v IIa  $p < 0.001$  for IIc v IIIc and Ic  $p < 0.005-0.001$  for VI v Id

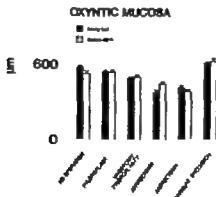


Fig 6 Height of the oxyntic mucosa in mm  $p < 0.001$  for IV and V v I  $p < 0.01$  for VI v I

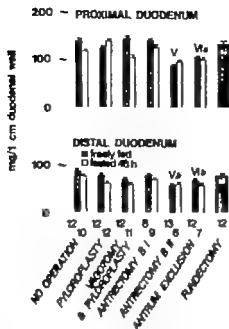


Fig 7 Weight in milligrams of 1 cm long specimens of the proximal (a) and distal (b) duodenal wall  $p < 0.005$   $\pm 0.01$  for  $V_a$  and  $VI_a$   $v$   $I_a$   $p < 0.005$   $0.001$  for  $V_b$  and  $VI_b$   $v$   $I$

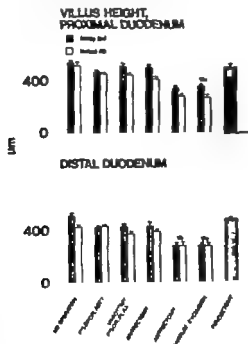


Fig 8 Villus height in  $\mu m$  in proximal (a) and distal (b) duodenal mucosa after the various operations  $p < 0.001$  for  $Vab$  and  $VIab$   $v$   $I$

was noted (Fig 8) Crypt depth was somewhat reduced following antrectomy BII but the effect was not statistically significant (Appendix: Table III)

The weight of small bowel mucosal scrapings (per unit length) of fasted and fed animals was unchanged after the various surgical procedures; the same applied to the weight of large bowel mucosal scrapings of fasted animals (Appendix: Table IV) In fed animals antrum exclusion but not fundectomy increased the weight of large bowel mucosal scrapings

The pancreas was heavier after antrum exclusion than after antrectomy (Fig 9)

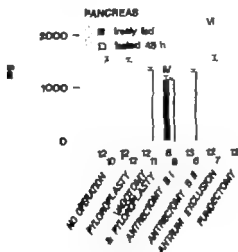


Fig 9 Weight of the pancreas after the various operations  
 $p < 0.005-0.001$  for IV  $\underline{v}$  I  $p < 0.001$  for VI  $\underline{v}$  I

**Nucleic acid and protein content** Changes in the content of DNA and RNA in the oxyntic mucosa induced by hyper- or hypogastrinemia could not be followed due to partial loss of the organ as a result of gastric surgery

In the small bowel mucosa no changes in DNA RNA or protein content were detected after the various gastric operations (Appendix: Tables V-VII) In the large bowel mucosa there was a tendency for the DNA and protein content to

increase after vagotomy and antrectomy BI

In the pancreas the protein content was increased by bypassing the duodenum (antrum exclusion and antrectomy BII) but nucleic acids and protein were not consistently affected by all other operations; i.e. changes in fed and fasted groups were not parallel (Appendix: Tables V-VII) Thus no correlation with the serum gastrin concentration was noted

#### DISCUSSION

The purpose of the present study was to establish whether the trophic effects of exogenous gastrin could be simulated by treatments that produced high serum concentrations of endogenous gastrin. The trophic effects of gastrin on oxyntic mucosa are widely accepted and there is much evidence that this is a physiologically important action (for recent reviews see Johnson 1976 1977). However the question remained: Would endogenous gastrin stimulate growth also of small and large bowel mucosa and of pancreas as claimed for exogenous gastrin? The various surgical interventions employed in the present study resulted in markedly different serum gastrin concentrations. Fundectomy greatly raised the serum gastrin concentrations: probably because of the high antral pH while antrectomy lowered the serum gastrin concentration. Vagotomy and antrum exclusion were less effective in raising the serum gastrin concentration. Although serum gastrin in the fasted state in these groups was elevated (probably as a result of high antral pH) this study gives no evidence that the serum gastrin levels in freely fed antrum excluded or vagotomized rats greatly exceeded those in control rats. It was therefore not clear whether a trophic effect was to be expected at all. However antrum exclusion and fundectomy produced a permanently raised serum gastrin concentration in contrast to the undulating serum gastrin concentration in freely fed unoperated rats. Not surprisingly therefore antrum exclusion increased the weight of the fundic pouch

( $p < 0.005$ ) and the height of the oxyntic mucosa ( $p < 0.01$ ) Antrectomy on the other hand lowered the height of the oxyntic mucosa ( $p < 0.001$ ) Thus perturbation of the serum gastrin concentration is accompanied by changes in the oxyntic mucosa

Also vagotomy raised the serum gastrin concentration The absence of a trophic effect of vagotomy on oxyntic mucosa a finding in keeping with earlier reports (Helander 1976 Crean et al 1969 Ley et al 1973) may be explained by a diminished responsiveness to gastrin perhaps because of an antitrophic effect elicited concomitantly Alternatively the concentration of circulating gastrin was too marginally increased to produce a trophic effect

Not even with the marked hypergastrinemia following fundectomy was a trophic effect seen in the remainder of the digestive tract Stimuli other than gastrin were effective however Thus the weight and villus height of the bypassed duodenum was greatly reduced after both antrum exclusion and antrectomy BII despite the fact that antrum exclusion produced hypergastrinemia and antrectomy hypogastrinemia By contrast the duodenum was unaltered after antrectomy BI and fundectomy Both these operations allow food to pass through the duodenum Hence intraluminal factors seem to be of great importance for the maintenance of the duodenal mucosa as proposed by Altmann (1974) Some workers (Mayston et al 1974 Mitznegg et al 1975) have argued in opposition to Johnson (1976 1977) that exogenous gastrin is without trophic effects on extragastric gut mucosa Our results suggest that this lack of effect applies also to endogenous gastrin (for a conflicting report see Mac Gregor and Way (1976)

The weight of the pancreas was increased after antrum exclusion and lowered after antrectomy BI These weight changes were accompanied by parallel changes in the RNA and protein content of the pancreas This is in agreement with the findings by Reber et al (1977) on rats subjected to



antrocolic transposition. However, since fundectomy and antrectomy BII did not change the pancreatic weight, the present results do not support the view that endogenous gastrin is important for regulating the size of the pancreas.

The results of the present study are in full agreement with the concept that endogenous gastrin exerts a trophic effect on the oxyntic mucosa. However, the magnitude of this effect is probably not determined by the serum gastrin concentration alone (see Hansen et al. 1976). The nervous activity of the gut wall and conceivably other intestinal hormones may modulate the response to gastrin; in the latter instance by additional trophic or antitrophic effects. The plasma somatostatin concentration was followed in a search for evidence that this hormone might be implicated. In the present study there was no evidence that somatostatin interfered with the trophic effects of gastrin.

It can be concluded from the present data that endogenous gastrin exerts a trophic effect on the oxyntic mucosa; however, there was no evidence for a similar effect of endogenous gastrin on the pancreas and extra-gastric gut.

# REFERENCES

- ALTMANN G G Demonstration of a morphological control mechanism in the small intestine Role of pancreatic secretions and bile In *Intestinal Adaptation*. Eds R H Dowling and M O Riecken F K Schattauer Verlag Stuttgart New York 1974 75-86
- ALUMETS J H A EL MUNSHID R HÄKANSON G LIEBERG J OSCARSON J P REHPELD and F SUNDLER Effect of antrum exclusion on endocrine cells of rat stomach *J Physiol (Lond.)* 1979 286 145-155
- ARIMURA A G LUNDQVIST J ROTHMANN R CHANG R FERNANDEZ-DE RANGO R ELDE D H COY C MEYERS and A V SCHALLY Radioimmunoassay of somatostatin *Metabolism* 1978 27 1139-1144
- ARNOLD H W CREUTZFELDT and J A HENNESSY Der pentagastirininduzierter Sauresekretion des Magens beim Menschen *Dtsch. Med. Wschr.* 1975 100 1014-1016
- BARROS D SA A A J S H BLOOM and J H BARON Direct inhibition of gastric acid by growth-hormone release-inhibiting hormone in dogs *Lancet* 1975 I 886-887
- BARROWMAN J A The trophic action of gastrointestinal hormones *Digestion* 1975 12 92-104
- BJÖRKLUND A B PALCK and CH ÖRSMAN Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic amines In *Methods of Investigative and Diagnostic Endocrinology* Editor S A Berson vol 1 *The Thyroid and Biogenic Amines* Editors J E Rall and S A Berson Amsterdam North Holland Publishing 1972 318-368
- BLOOM H R CH MORTIMER M O THORNER S M BESSER R HALL A GOMEZ-PAN V M ROY R C G RUSSELL D H COY A J KASTIN and A V SCHALLY Inhibition of gastrin and gastric acid secretion by growth hormone release-inhibiting hormone *Lancet* 1974 I 1106-1109
- CAPOFERRO R and K NYGAARD Effects of antrectomy on the gastric mucosa of the rat *Scand. J Gastroenterol* 1973 8 347-352
- CASTELEYN P P M DUBRASQUET and G WILLEMS Opposite effects of gastrin on cell proliferation in the antrum and other parts of the upper-gastrointestinal tract in the rat *Am. J. Dig. Dis.* 1977 22 798-804
- CREAN G P A A GUNN and D E RUMSEY The effects of vagotomy on the gastric mucosa of the rat *Scand. J Gastroenterol* 1969 4 675-680
- GOMEZ-PAN A M ALBINUS J H REED H SHAW H HALL G M BESSER D H COY A J KASTIN and A V SCHALLY Direct inhibition of gastrin and pepsin secretion by growth hormone release-inhibiting hormone in cats *Lancet* 1975 I 888-890

- HAKANSON R L -I LARSSON G LIEDBERG J OSCARSON P  
SUNDLER and J VANG Effects of antrectomy or portacaval  
shunting on the histamine-storing endocrine-like cells in  
oxyntic mucosa A fluorescence histochemical electron  
microscopic and chemical study *J Physiol (Lond)* 1976  
785-800
- HANSEN O H T PEDERSEN J K LARSEN and J F REHFELD  
Effect of gastrin on gastric mucosal cell proliferation in  
man *Gut* 1976 17 536-541
- HELANDER H F Stereological changes in rat parietal cells  
after vagotomy and antrectomy *Gastroenterology* 1976 71  
1010-1018
- HINRICHS H R R O PETERSEN and R BASERGA Incorporation  
of thymidine into DNA of mouse organs *Arch Patol* 1964 78  
245-253
- JOHNSON L R The trophic action of gastrointestinal hor-  
mones *Gastroenterology* 1976 70 278-288
- JOHNSON L R New aspects of the trophic action of gastro-  
intestinal hormones *Gastroenterology* 1977 72 788-792
- JOHNSON L R and M I GROSEMAN Characteristics of inhibi-  
tion of gastric secretion by secretin *Am J Physiol* 1969  
217 1401-1404
- JOHNSON L R and D GUTHRIE Effect of cholecystokinin and  
16 16-dimethyl prostaglandin E<sub>2</sub> on rat RNA and DNA of  
gastric and duodenal mucosa *Gastroenterology* 1976 70 59-65
- JOHNSON L R L M LICHTENBERGER and E M COPELAND Action  
of gastrin on gastrointestinal structure and function  
*Gastroenterology* 1975 68 1184-1192
- KONTUREK S J J TASLER M CIESIOWSKI D H COY and A V  
SCHALLY Effect of growth hormone release-inhibiting hor-  
mone on gastric secretion mucosal blood flow and serum  
gastrin *Gastroenterology* 1976 70 737-741
- LEHY C R A BOHNEFOND R DUBRASQUET S NASCA M LEWIN  
and B BONFELS Comparative effects of antrocolic trans-  
position and antrectomy on fundic mucosa and acid secretion  
of the rat *Gastroenterology* 1973 64 421-428
- LEY R G WILLEMS and Y VANSTEENKISTE Influence of vago-  
tomy on parietal cell kinetics in the rat gastric mucosa  
*Gastroenterology* 1973 65 764-772
- LOWRY O H N J ROSEBROUGH A L FARR and N J RANDALL  
Protein measurement with the Folin phenol reagent *J Biol  
Chem* 1951 193 265-275
- LUNDQVIST G E GUSTAVSSON E ELDE and A ARIMURA A radio-  
immunosorbent assay for plasma somatostatin *Clin Chim Acta*  
In press 1979
- MAC GREGOR I L and L W WAY Chronic hypergastrinemia pro-  
duces hypertrophy of the liver and intestine in rats *Surg  
Forum* 1976 27 411-413
- MAK K M and W W L CHANG Pentagastrin stimulates epitheli-  
al cell proliferation in duodenal and colonic crypts in  
fasted rats *Gastroenterology* 1976 71 1117-1120

- MANSBACH C M R M WILKINS W O DOBBINS and M P TYOR  
Intestinal mucosal function and structure in the steatorrhea of Zollinger-Ellison syndrome *Arch Intern Med* 1968 121 487-498
- MARTIN F I B MACLEOD and W SIRCUS Effects of antrectomy on the fundic mucosa of the rat *Gastroenterology* 1970 59 437-444
- MAYSTON P D and J A BARROWMAN Influence of chronic administration of pentagastrin on the pancreas in hypophysectomized rats *Gastroenterology* 1973 65 391-399
- MAYSTON P D J A BARROWMAN and R H DOWLING Effect of pentagastrin on small bowel structure and function in the rat *Digestion* 1974 12 78-84
- MITZNEGG P W DOMSCHKE S DOMSCHKE D BELOHLAVEK W SPRÜGEL U STRUBB E WUNSCH E JAEGER and L DEMLING  
Protein synthesis in human gastric mucosa: Effects of pentagastrin secretin and l3-nle-motilin *Acta Hepato-Gastroenterol* 1975 22 333-335
- MORDES J P M HERRERA and W SILEN Decreased weight gain and food intake in vagotomized rats *Proc Soc Exp Biol Med* 1977 156 257-260
- NEUBURGER PH M LEWIN C and S BONFILS  
Parietal and chief cell populations in four cases of the Zollinger-Ellison syndrome *Gastroenterology* 1972 63 937-942
- OSCARSON J E A H F VEEN J S ROSS and R A MALT  
Dimethylhydrazide induced colonic carcinoma in the rat: Dissociation from endogenous gastrin levels / Submitted to *Cancer Res*
- OSCARSON J E A H F VEEN R C N WILLIAMSON J S ROSS and R A MALT  
Compensatory hyperplasia and starvation atrophy in small bowel: Dissociation from endogenous gastrin levels *Gastroenterology* 1977 72 890-895
- PETERSEN H T E SOLOMON and M I GROSSMAN Effect of chronic pentagastrin cholecystokinin and secretin on pancreas of rats *Amer J Physiol* 1978 234 286-293
- PHILLIP J S DOMSCHKE W DOMSCHKE H J URBACH M REISS and L DEMLING Inhibition by somatostatin of gastrin release and gastric acid responses to meals and to pentagastrin in man *Scand J Gastroenterol* 1977 12 261-265
- REBER H A F JOHNSON K DEVENEY C MONTGOMERY and L W MAY  
Trophic effects of gastrin on the exocrine pancreas in rats *J Surg Res* 1977 22 554-560
- REHFELD J F and F STADIL Gel filtration studies on immunoreactive gastrin in sera from Zollinger-Ellison patients *Gut* 1973 14 369-373
- SCOTT J F A P FRACCASTORO and E B TAFT Studies in histochemistry: I Determination of nucleic acids in microgram amounts of tissue *J Histochem Cytochem* 1956 4 1-10
- SHIMODA S E D R SAUNDERS and E E RUBIN The Zollinger-Ellison syndrome with steatorrhea II The mechanisms of fat and vitamin B<sub>12</sub> malabsorption *Gastroenterology* 1968 55 705-723

- STADIL F and J F REHFELD Radioimmunoassay of gastrin in human serum *Scand J Gastroenterol* 1971 suppl 9 61-65
- STADIL F and J F REHFELD Preparation of  $^{125}\text{I}$ -labelled synthetic human gastrin I for radioimmunoanalysis *Scand J Clin Lab Invest* 1972 30 361-368
- STADIL F and J F REHFELD Determination of gastrin in serum An evaluation of the reliability of a radioimmunoassay *Scand J Gastroenterol* 1973 8 101-112
- STANLEY M H R E COALSON M I GROSSMAN and L H JOHNSON Influence of secretin and pentagastrin on acid secretion and parietal cell number in rats *Gastroenterology* 1972 63 264-269
- VATN M H E SCHRUMPF K F HANSEN and J MYREN The effect of somatostatin on pentagastrin-stimulated gastric secretion and on plasma gastrin in man *Scand J Gastroenterol* 1977 12 833-839
- WIDE L Radioimmunoassay employing immunosorbents (*Acta Endocr (Kbh)*) 1969 suppl 174 207-221
- WILLEMS M W GEPTS and A BREMER Endogenous hypergastrinemia and cell proliferation in the fundic mucosa in dogs *Am J Dig Dis* 1977 22 419-423
- WISEMAN D A and L R JOHNSON Evidence that secretin does not have direct antitrophic effect on the rat stomach *Proc Soc Exptl Biol Med* 1976 153 277-279

# APPENDIX

Variations in serum concentration: Trophic effect on the gastro-intestinal tract of the rat

J Oscarson R Håkanson G Liedberg G Lundqvist P Sundler and J Thorell

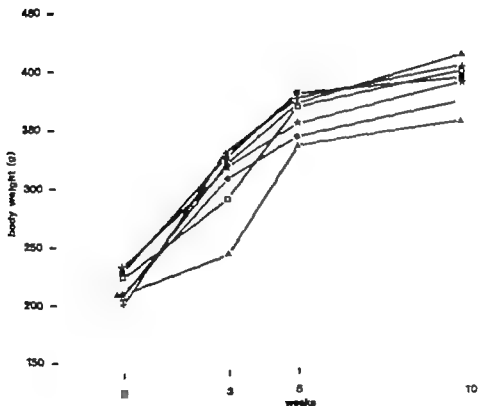


Fig. 1. Weight development during the 10 postoperative weeks. Pyloroplasty  $\Delta$ , vagotomy and pyloroplasty  $\triangle$ , antrectomy  $\square$ , antrectomy  $\blacksquare$ , antrectomy and vagotomy  $\circ$ , antrectomy and vagotomy  $\otimes$ , antrectomy and vagotomy  $\oplus$ , antrectomy and vagotomy  $\ominus$ . The group  $\ominus$  with no operation  $(\circ)$  is also included.

Table I Survival rates following the various operations

Surgical treatment	Survival/total number	Survival per cent
No operation	22/22	100
Pyloroplasty	24/24	100
Vagotomy and pyloroplasty	23/24	96
Antrectomy B I	17/32	53
Antrectomy B II	19/32	59
Antrum exclusion	19/33	58
Fundectomy	12/12	100

Table II Concentration of somatostatin in plasma

Surgical treatment	Freely fed		Fasted	
	pg/ml	mean $\pm$ S.E.	(n)	
No operation	367 $\pm$ 35.3	(12)	146 $\pm$ 30.0 <sup>+++</sup>	(4)
Pyloroplasty	285 $\pm$ 38.9	(12)	190 $\pm$ 21.0 <sup>†</sup>	(6)
Vagotomy	261 $\pm$ 35.5	(10)	228 $\pm$ 17.1	(6)
Antrectomy B I	198 <sup>xxx†</sup>	4.6 (6)	134 $\pm$ 20.2 <sup>††</sup>	(5)
Antrectomy B II	223 <sup>xxx†</sup>	21.6 (12)	223 $\pm$ 45.7	(3)
Antrum exclusion	305 $\pm$ 22.9	(12)	234 $\pm$ 22.1 <sup>†</sup>	(4)
Fundectomy	215 <sup>xxx</sup>	15.9 (12)	-	

Statistical significance is expressed throughout by x or + for  $0.01 < p < 0.05$  xx or ++ for  $0.001 < p < 0.01$  and xxx or +++ for  $p < 0.001$ . The symbol x denotes the difference between control rats and operated rats (within one vertical row). The symbol + denotes the difference between freely fed and fasted rats (within one horizontal row). No operation is control for pyloroplasty. Antrectomy B I and B II, antrum exclusion and fundectomy. Pyloroplasty is control for vagotomy.

Table III Crypt depth of the proximal and distal duodenal mucosa after the various surgical procedures<sup>a</sup>

	Crypt depth proximal duodenum		Crypt depth distal duodenum	
	Freely fed	Fasted	Freely fed	Fasted
No operation	212 $\pm$ 8 (12)	210 $\pm$ 16 (7)	204 $\pm$ 8 (12)	174 $\pm$ 10 (8)
Pyloroplasty	202 $\pm$ 8 (12)	198 $\pm$ 6 (12)	182 $\pm$ 4 (11)	176 $\pm$ 8 (12)
Vagotomy and pyloroplasty	202 $\pm$ 8 (12)	176 $\pm$ 6 (10)	164 $\pm$ 8 (12)	158 $\pm$ 8 (10)
Antrectomy B I	214 $\pm$ 8 (10)	200 $\pm$ 14 (9)	186 $\pm$ 8 (8)	168 $\pm$ 10 (8)
Antrectomy B II	182 $\pm$ 10 (13)	170 $\pm$ 6 (6)	180 $\pm$ 6 (13)	170 $\pm$ 8 (6)
Antrum exclusion	200 $\pm$ 8 (13)	178 $\pm$ 12 (8)	185 $\pm$ 10 (13)	164 $\pm$ 6 (7)
Fundectomy	220 $\pm$ 8 (12)	-	198 $\pm$ 7 (12)	-

<sup>a</sup> Expressed as pm mean  $\pm$  S E (n)



Table IV Weight of the small and large bowel mucosa <sup>a</sup>

	Small bowel		Large bowel	
	Freely fed	Fasted	Freely fed	Fasted
No operation	193 ± 8 (12)	140 ± 6 <sup>†††</sup> (10)	236 ± 10	190 ± 16 <sup>†</sup>
Pyloroplasty	191 ± 9 (12)	165 ± 6 <sup>†</sup> (12)	232 ± 6	258 ± 16
Vagotomy and pyloroplasty	211 ± 7 (12)	147 ± 5 <sup>†††</sup> (11)	204 ± 8	292 ± 24 <sup>††</sup>
Antrectomy B I	213 ± 14 (8)	152 ± 8 <sup>††</sup> (9)	220 ± 20	303 ± 24 <sup>†</sup>
Antrectomy B II	196 ± 7 (13)	166 ± 8 <sup>†</sup> (6)	261 ± 20	240 ± 18
Antrum excision	235 <sup>xx</sup> ± 15 (22)	276 ± 11 <sup>††</sup> (7)	328 <sup>xx</sup> ± 14	227 ± 27 <sup>††</sup>
Fundectomy	196 ± 10 (12)	-	267 ± 14	-

<sup>a</sup> Expressed as milligrams of mucosal scrapings from 3 cm long specimens

Tabl V DMA cont t i small and large bowel mucosa and pancreas

	Small bowel			Large bowel			Pancreas	
	Freely fed ( )	F	tad ( )	Freely fed	Fasted		Freely fed	Fasted
No operation	1 7 0 1 (12)	1 1	0 1 <sup>†††</sup> (10)	2 2 + 0 1	1 5 + 0 2 <sup>††</sup>		26	2 18 + 2
Pyl roplasty	1 7 0 1 (12)	1 3	0 1 <sup>†</sup> (12)	2 3 + 0 1	2 2 <sup>xxx</sup> 0 1		22	+ 2 16 + 1
Vagotomy and Pyl roplasty	1 6 + 0 1 (12)	1 0	0 0 4 <sup>†††</sup> (11)	1 8 <sup>xxx</sup> 0 1	2 0 0 2		25	+ 2 25 <sup>xxx</sup> 2
Anrectomy B I	1 6 0 2 (8)	1 1	0 1 <sup>††</sup> (9)	1 6 + 0 2	3 4 <sup>xxx</sup> + 0 2 <sup>†††</sup>		11 <sup>xxxx</sup> 2	22 ± 2 <sup>††</sup>
Anrectomy B II	1 6 0 1 (13)	1 3	0 2 (6)	2 4 + 0 2	2 1 + 0 3		16 <sup>xxxx</sup> 1	21 2
Antrum occlusion	1 8 0 1 (12)	1 2	0 1 <sup>†††</sup> (7)	2 5 ± 0 3	1 7 - 0 3		22 + 1	38 <sup>xxxx</sup> 3 <sup>†††</sup>
Fundectomy	2 9 ± 0 1 (12)			2 6 0 3			16 <sup>z</sup>	2

a Expressed as g per 5 cm mucosa (small and large bowel) x mm pancreas

Tabl 11 RNA content in small and l rye bowel and pancreas

	Small bowel		Large bowel		Pancreas	
	Freely f d ( )	Fasted ( )	Freely fed	Y sted	Freely fed	Fasted
Mo ope tion	2 0 0 2 (12)	1 2 0 2 <sup>†</sup> (10)	2 1 0 3	1 8 0 2	75	4 53 3 <sup>††</sup>
Pyl ropi ty	2 2 0 2 (12)	1 5 0 2 <sup>†</sup> (12)	2 1 0 2	2 3 0 3	59	4 57 4
Vagotomy and pyl ropi ty	1 6 0 2 (12)	1 1 0 1 <sup>††</sup> (11)	1 8 0 2	1 6 0 1	61	3 87 4
Antrectomy B I	2 2 0 4 ( 7)	1 3 0 2 ( 9)	1 8 0 2	2 9 <sup>xxx</sup> 0 3 <sup>††</sup>	49 <sup>xxx</sup>	4 50 3
Antr ectomy B II	1 8 0 1 (13)	1 1 0 3 <sup>†</sup> ( 6)	2 8 0 3	1 9 0 3 <sup>†</sup>	54 <sup>xxx</sup>	3 57 4
Astrum exlusion	2 2 0 1 (12)	1 3 0 3 <sup>†</sup> ( 7)	2 8 0 3	2 5 0 3	77	3 108 <sup>xxx</sup> 6 <sup>††</sup>
Pancreatectomy	1 8 0 2 (12)		2 5 0 2		76	5

Expressed as mg pe 5 cm mucosa (small and large bowel) or per pancreas

Tabl VII Prot in content in small and larg bowel and pancreas \*

	Small bowel			Large bowel			Pancreas	
	Freely fed ( )	Pasted (n)		Freely fed	Pasted		Freely fed	Pasted
No op e tion	34 2 (12)	18 3 <sup>+++</sup> (10)		38 3	30 + 3		290 + 19	293 13
Pyl ropla ty	30 2 (12)	22 3 <sup>+</sup> (12)		40 + 4	28 3		299 $\pm$ 6	338 <sup>±</sup> $\pm$ 9 <sup>++</sup>
Vegetomy and pyloroplasty	34 3 (12)	20 2 <sup>+++</sup> (11)		28 3	46 <sup>xxx</sup> 3 <sup>+++</sup>		290 9	337 <sup>x</sup> 9 <sup>++</sup>
Astractory B I	34 3 ( 8)	27 4 ( 9)		33 + 4	57 <sup>xxx+</sup> 4 <sup>+++</sup>		225 <sup>xx</sup> 7	280 - 9 <sup>+++</sup>
Astractory B II	34 3 (13)	27 5 ( 6)		42 + 4	34 5		357 <sup>xxx</sup> + 8	349 <sup>xx</sup> + 11
Antrum exclusion	38 2 (12)	35 6 ( 7)		36 5	40 + 6		514 <sup>xxx</sup> 12	347 <sup>xx</sup> + 10 <sup>+++</sup>
Pancrectomy	27 3 (12)			32 5			269 7	

Expre seed 20g per 5 cm mucosa (small and large bowel) or per pancrea



**ACTA PHYSIOLOGICA SCANDINAVICA**  
**Supplementum 476**

**DYNAMIC AND STATIC COMPONENTS  
IN THE MYOGENIC CONTROL OF VASCULAR TONE  
IN CAT SKELETAL MUSCLE**

**BY**  
**PER-OLOF GRÄNDE**

**LUND 1979**



**ACTA PHYSIOLOGICA SCANDINAVICA**

**Supplementum 476**

**From the Institute of Physiology and Biophysics**

**University of Lund**

**Sweden**

**DYNAMIC AND STATIC COMPONENTS**

**IN THE MYOGENIC CONTROL OF VASCULAR TONE**

**IN CAT SKELETAL MUSCLE**

**BY**

**PER-OLOF GRÄNDE**

**LUND 1979**





## CONTENTS

INTRODUCTION	5
Previous concepts of myogenic vascular control	5
Aim of the present investigations	10
METHODOLOGICAL CONSIDERATIONS	12
Experimental preparation	12
Recordings of total and segmental vascular resistances in the muscle region	13
DESIGN OF AN ELECTRONIC DIFFERENTIAL PRESSURE FLOWMETER AND A RESIS- TANCE METER FOR CONTINUOUS MEASUREMENT OF VASCULAR RESISTANCE	15
RESULTS AND COMMENTS	18
Static and dynamic myogenic reactivity in the vascular bed of skeletal muscle	18
Role of intra-arterial static and dynamic transmural pressure sti- muli in the development of normal basal vascular tone	24
Adrenergic influences on myogenic microvascular reactivity in skeletal muscle	27
GENERAL DISCUSSION AND SUMMARY	31
SUMMARY	38
ACKNOWLEDGEMENTS	40
REFERENCES	41

This summary is based on the following publications:

- I GRÖNDE P O & BORGSTRÖM P 1978 An electronic differential pressure flowmeter and a resistance meter for continuous measurement of vascular resistance Acta Physiol Scand 102:224-230
- II GRÖNDE P O LUNDVALL J & MELLANDER S 1977 Evidence for a rate sensitive regulatory mechanism in myogenic microvascular control Acta Physiol Scand 99:432-447
- III GRÖNDE P O & MELLANDER S 1978 Characteristics of static and dynamic regulatory mechanisms in myogenic microvascular control Acta Physiol Scand 102:231-245
- IV GRÖNDE P O BORGSTRÖM P & MELLANDER S On the nature of basal vascular tone in cat skeletal muscle and its dependence on transmural pressure stimuli Acta Physiol Scand in press
- V GRÖNDE P O & MELLANDER S 1979 Beta adrenergic inhibitory interference with myogenic vascular reactivity during experimental intervention Acta Physiol Scand 106:87-89
- VI GRÖNDE P O 1979 Influence of neural and humoral beta adrenoceptor stimulation on dynamic myogenic microvascular reactivity in cat skeletal muscle Acta Physiol Scand in press

In the text these papers are referred to by their Roman numerals

The maintenance of cardiovascular homeostasis in normal life and in pathophysiological stress situations implies continuous adjustments of cardiac performance and vascular tone so as to satisfy the highly variable circulatory demands of the different tissues. The regulation of the peripheral vascular functions is accomplished by adjustments of excitatory and inhibitory influences on vascular tone in the various circuits of the circulatory system. Such alterations of smooth muscle tone are effected by regulatory mechanisms originating from sites within and outside the tissue itself, i.e. via the local and central vascular control systems. Although details of integrated central control remain to be clarified, several of the specific mediators, the neurotransmitters and the circulating hormones, have been identified unequivocally. Knowledge about the final links of the local control systems is much less complete. In particular with regard to the so-called myogenic control system. The present investigations serve to elucidate in some detail the receptor-effector characteristics of myogenic control and its mode of operation in the vascular bed of skeletal muscle.

#### Previous concepts of myogenic vascular control

In 1902 Bayliss put forward his classical hypothesis that the intravascular blood pressure via distension caused a direct stimulating action on the vascular smooth muscle and enhanced contractility contributing to resting vascular tone and its regulation. The hypothesis was based on a series of careful though not entirely conclusive experiments and on analogies to the responses to stretch of other types of smooth muscle. Bayliss concluded that these responses were of myogenic origin and the term myogenic reactivity has later by convention been used to denote the ability of the vascular smooth muscle to respond to the mechanical force exerted by the blood pressure. Although the term myogenic is a relatively non-specific classification of this particular response (which in fact might have contributed to some confusion in this field of research) this terminology is so commonly used that it was adopted also in the present series of papers.

The myogenic hypothesis of Bayliss was seriously questioned for several decades starting with a critical study by Anrep (1912) who claimed that Bayliss' experimental results could be explained by other mechanisms. In particular by

local metabolic influence. The opinion was then quite commonly maintained for considerable length of time that variation in tissue metabolite concentration was the principal means for local regulation of vascular tone. An apparent obstacle for further progress in this field of research was the considerable difficulties encountered in designing specific tests of the myogenic hypothesis without simultaneous experimental interference with the local metabolic vascular control system; another problem is the apparent vulnerability of the myogenic control system to experimental intervention (see below).

The myogenic hypothesis was however revived by later hemodynamic studies. Especially important was the work by Folkow in 1949, who quite convincingly could demonstrate active myogenic reactions to changes in blood pressure under circumstances of relatively minor metabolic influence. This investigation led to a great many other more detailed hemodynamic whole organ and vital microscopy studies in various tissues which further supported the myogenic hypothesis (e.g. Folkow and Öberg 1961, Hellander, Öberg and Odellram 1964, Johnson and Wayland 1967, Johnson 1968, Baez, Laidlaw and Orkin 1974) and with time to the now quite generally accepted compromise view that metabolic as well as myogenic mechanisms are important links in local vascular control and synergistically responsible for autoregulation of blood flow (for ref. see Johnson 1964, Hellander and Johansson 1968).

Myogenic mechanisms also seem to be involved in other autoregulatory phenomena. It was thus demonstrated by Folkow and Öberg (1961) and especially by Jörhult and Hellander (1974) that there is an effective myogenic autoregulation of capillary hydrostatic pressure in skeletal muscle over a wide range of arterial pressures from 30 up to 170 mm Hg, serving to maintain the normal transcapillary Starling fluid equilibrium during arterial hypo- and hypertension. Hellander, Öberg and Odellram (1964) demonstrated that increased vascular transmural pressure as occurs in dependent vascular beds during hydrostatic load caused an especially marked myogenic constriction of the precapillary sphincters in skeletal muscle and skin tissues. This study led to the concept of myogenic autoregulation of transcapillary filtration during increased transmural pressure which effectively could prevent the theoretically expected oedema formation. These whole organ studies strongly pointed to the fact that myogenic reactions were especially vivid in the arterial microvessels. This view was later confirmed more directly by vital microscopy observations in the microcirculation (e.g. Johnson 1968, Baez, Laidlaw and Orkin 1974) which demonstrated that increased vascular transmural pressure as all

cited by raised venous pressure. Indeed caused the predicted myogenic diameter changes of the small precapillary vessels. Johnson and Wayland (1967) also showed distinct dependence of capillary flow periodicity and myogenic vasomotion upon change of intravascular pressure.

Although all these studies provided strong support for the myogenic hypothesis it has not been universally accepted. An alternative interpretation of the active constrictor response to raised vascular transmural pressure was put forward by Gaskell and Burton (1953) and Haddy and Gilbert (1956) who attributed this response to a local nervous veno-arteriolar reflex mechanism. This explanation however was questioned on the basis of histological examinations which gave no evidence for local nerve plexus in the vascular wall and further on the basis of preserved responses after adrenergic blockade (for ref. see Folkow 1962). Yet such a veno-arteriolar reflex might exist in some vessels as suggested by recent expts. on the vascular bed of adipose tissue in the human (Henriksen 1976) and may here operate in synergism with the myogenic mechanism during a transmural pressure change.

Some authors (e.g. Guyton 1964) have more or less rejected the myogenic hypothesis on the basis of a theoretical reasoning that myogenic control implies a positive feedback that could lead to extreme myogenic effector reactions and to serious instability in vascular regulation. Further it has been stated that myogenic control could be self-limiting insofar that a myogenic smooth muscle shortening in response to raised vascular distending pressure automatically would abolish the primary receptor stimulus if the receptors were sensitive to change in length (Gaskell and Burton 1953).

Arguments against the above-mentioned objections have been derived from knowledge of vascular smooth muscle physiology from circulatory studies and from mathematical models of myogenic control. A tentative description of the myogenic hypothesis was thus put forward by Folkow (1964) which implied that vascular distension increases the frequency of rhythmic contractions initiated by pacemaker cells and propagated to adjacent smooth muscle cells. A static elongation beyond control length of the myogenic sensor element during the period of relaxation of the smooth muscle would then occur between repetitive myogenic contractions, the latter causing enhanced resistance to flow. Another explanation was given by Johnson and Wayland (1967) who postulated that the myogenic sensor element was coupled in series with the contractile units of the vasculature and that the myogenic reactions might be related to changes of wall tension.

It is obvious, that the exact events are difficult to predict from in vivo observations due to the complicating effects of Laplace and Poiseuille's laws and of wall/lumen variations on wall tension. A recent mathematical model study of myogenic vascular control which took such factors into account (Borgström and Grände 1979) can aid in our understanding of those problems. Such mathematical analysis provided more direct support for the opinion that myogenic reactions are triggered by and related to change of wall tension. It further contradicted the objection that myogenic control via positive feedback necessarily would imply instability in vascular regulation and predicted instead that extreme myogenic effector reactions would not occur in response to changed vascular transmural pressure. Possible extreme effects of positive feedback in myogenic control would further be limited simply by the restricted upper frequency range of the myogenic pacemakers (Johansson and Mellander 1975) and also by the dilator influence of metabolites accumulating as a result of flow restriction during myogenic constriction (Berne 1964).

The vast majority of studies in this field of research thus provide strong evidence for Bayliss' hypothesis, and the myogenic mechanism is now quite widely appreciated as an important factor in the development of active responses to changes in blood pressure. This in turn implies that such a myogenic mechanism acts by increasing or decreasing the normal basal vascular tone. Basal vascular tone in this context refers to the marked contractile state which at normal pressure remains on the arterial side of most vascular beds after elimination of known nervous and humoral excitatory influences (e.g. Löfving and Mellander 1956; Folkow 1962).

Various explanations for the basal vascular tone have been proposed. For instance it has been postulated by Johansson and Bohr (1966) that there is a plasma factor which is responsible for the tonic activity in the small blood vessels but the evidence is so far incomplete and the nature of the factor still unknown. It has also been suggested that oxygen acting directly on the vascular smooth muscle causes the normal vascular contractile state (Ross, Fairchild, Weddy and Guyton 1962; Guyton et al. 1964) but more likely oxygen is just supporting contraction at basal tone rather than initiating it (cf. Duling 1972). There is much to indicate however that basal vascular tone is of intrinsic nature (Folkow 1962) and in some way closely related to the above described myogenic reactivity. Basal tone might then result from spontaneous myogenic automaticity in the vascular smooth muscle, but it might also require some specific continuing stimulus for its development and maintenance.

for instance initiated by the arterial blood pressure distension as apparently envisaged by Bayliss. Basal tone might even be established by somewhat different mechanisms in the various sections of the vascular bed in view of the documented heterogeneity of vascular smooth muscle in different parts of the circulatory system (e.g. Bohr 1965, Vanhoutte 1978). It is apparent that these hypothetical explanations of basal myogenic tone require further clarification.

Some recent studies have added new dimensions to the concept of myogenic vascular reactivity by description of the electrophysiological events and of a seemingly important rate sensitivity in myogenic control.

In the previous studies myogenic vascular reactivity was analysed almost exclusively in terms of the active responses which develop upon a given steady state increase or decrease in vascular transmural pressure. Such steady-state myogenic effector reactions might at least a priori be considered to be the result of a static stimulus related to the amplitude of the transmural pressure change. Some indication for the presence of a rate-sensitive or dynamic component in myogenic vascular control was first obtained from studies of the mechanical vascular response to stretch (Sparks 1964) and from experimental and theoretical analyses of amplitude and frequency characteristics of pressure induced changes of flow resistance in the renal and coronary circulations (Basar and Weiss 1969) and from observations of increased vascular tone upon shift from steady to normal pulsatile pressure perfusion in skeletal muscle (Mellander and Arvidsson 1974).

Direct evidence for an important dynamic component in the myogenic vascular response to passive change in length was more recently obtained from in vitro observations on the single-unit smooth muscle of the rat portal vein (Johansson and Mellander 1975) a study which also elucidated the underlying electrophysiological mechanisms. The vascular smooth muscle preparation in this investigation was exposed to a given passive static stretch or shortening applied at various rates of length ( $L$ ) change  $dL/dt$  over the range between -3 and +3 % of the muscle length/s. Static stretch by 40 % of the muscle length was shown to evoke only moderate excitatory effects in terms of a 10-15 % increase in spike discharge above the resting control value and a corresponding increase in active force. Dynamic stretch during the period of increasing muscle length was much more effective causing graded excitatory responses which at high  $dL/dt$  values were up to 15-20 times greater than those evoked by static stretch. Passive shortening at different rates caused graded inhibitory responses.



ses below the control level: complete inhibition of electrical and mechanical activity being observed at the highest negative test value of  $dL/dt$ . Further analysis suggest that the dynamic myogenic response was more closely correlated to the rate of change of passive tension ( $da/dt$ ) than to  $dL/dt$  (Sigurdsson, Johansson and Hellander 1977).

These in vitro studies which seem to be the first direct electrophysiological demonstrations of myogenic vascular reactivity provided direct support for the view previously tentatively discussed by Folkow (1964) that the myogenically active vascular smooth muscle can be considered to act as a mechanoreceptor-mechanoeffector unit in which distension via effects on a pacemaker mechanism causes facilitation of spike-discharge propagated to the neighbouring smooth muscle effector cells. The net effect of such mechano-electrical coupling in response to receptor deformation is changes in the frequency of spike generation and attendant active changes in mechanical activity or vascular tone. The in vitro studies also made it clear that myogenic reactivity in the portal vein is dependent not only on the static characteristics of the pacemaker stimulus but also and more distinctly on its dynamic characteristics.

#### Aim of the present investigations

The studies summarized in this paper deal with the myogenic control of the vascular bed in cat skeletal muscle and in particular with the problem whether such regulation is characterized by rate-sensitivity i.e. by a dynamic component in addition to a static component as previously demonstrated for myogenic reactivity in the rat portal vein in vitro (Johansson and Hellander 1975). Both these components in myogenic control were shown to exist in the studied vascular bed (lower hind leg muscles). The papers will describe in some detail the localization of these components, their receptor-effector characteristics and their possible functional significance for myogenic control in general and for the initiation and maintenance of normal basal vascular tone. An analysis of the interaction between myogenic and adrenergic control in the muscle circulation was also performed.

The expts. were designed so as to permit analysis of static and dynamic myogenic reactivity to transmural pressure stimuli under conditions of almost negligible metabolic influence. This was accomplished by changing vascular transmural pressure in the muscle region via alteration of extravascular pressure thereby avoiding the quite large passive flow changes inherent in previous approaches in which arterial or venous pressure was altered.

A differential pressure flowmeter and a resistance meter were constructed (paper I) to permit continuous and accurate recordings of arterial blood flow and of total resistance as well as segmental resistances in proximal arterial vessels, microvessels and large veins in the vascular bed of skeletal muscle.

Evidence for the existence of a static and a rate sensitive dynamic component in myogenic vascular control was presented in paper II. Dynamic reactivity was here shown to be localized to microvessels smaller than about 20 to 25  $\mu\text{m}$  (I d.) on the arterial side.

Paper III describes in greater detail the stimulus effector characteristics and the possible functional significance of the static and the dynamic components in myogenic microvascular control in skeletal muscle.

In paper IV an attempt was made to elucidate the nature of the basal tone in various segments of the muscle vascular bed by determining its dependence on normal arterial blood pressure via static mean pressure distension and dynamic pulse pressure distension as well as its responsiveness to strong inhibitory transaural pressure stimuli. The analysis suggested that the nature of basal tone is different in large compared to small arterial vessels.

It is a well known fact that myogenic reactivity can be severely depressed by experimental intervention. Expts. in paper V demonstrated that this unexplained phenomenon to a major extent could be attributed to a  $\beta$  adrenergic inhibitory effect on myogenic reactivity exerted by catecholamine release from the adrenals.

The interaction between adrenergic and myogenic regulatory mechanisms was analysed more systematically in paper VI which revealed that activation within the physiological range of the sympatho-adrenal  $\beta$  adrenergic vascular control system can modify myogenic reactivity significantly and in a manner which seems to refine integrated vascular regulation.

### Experimental preparation

The expts were performed on a total of 73 cats (b wt 2.8 - 5.0 kg) after anaesthesia with  $\alpha$ -chloralose and urethane. Body temperature was kept at  $38 \pm 0.5^\circ\text{C}$ .

All observations were made on the acutely denervated vascular bed of the lower leg muscles of the cat hind limb. The surgical preparation was similar to that previously described by Kjellmer (1964) with the skinned muscle region separated from the body except for its main vascular connections: the popliteal artery and vein.

After heparinization, arterial blood flow was diverted from the femoral to the popliteal artery via a shunt circuit with a T-tube connection which permitted measurements of arterial inflow pressure (AP) and close arterial administration of drugs. Venous outflow was shunted from the popliteal to the external jugular vein and venous outflow pressure (VP) was monitored from a T-tube close to the muscle region. In the majority of expts, regional blood flow was measured on the arterial side with a specially designed pressure gradient flowmeter (see next chapter) which was found to be superior to an electromagnetic flowmeter or a venous drop recorder unit used in some of the early expts. The muscle region was autoperfused from the animal in all expts except for those described in paper VI and in one series of expts in paper IV where perfusion instead was accomplished with the aid of a pressure servo-controlled roller pump inserted in the arterial shunt circuit. This pump could deliver normal pulsatile or non-pulsatile flows. Arterial inflow pressure could be adjusted to desired levels with a screw clamp placed around the arterial shunt circuit (paper IV). An inert material (silicone rubber) was used for all tubes in contact with blood (shunts, flowmeter, perfusion pump) creating an entirely smooth inner surface of the tubings.

To be able to follow not only total vascular resistance in the muscle region but also segmental resistances, pressures were monitored from two additional sites along the vascular tree from artery to vein: viz. from arterial microvessels (small artery pressure - SAP) and from venous microvessels (small vein pressure - SVP). These pressures were measured via small catheters inserted in

the sural artery and vein according to a technique modified from that originally described by Raddy et al (1954) and later used in several studies (e.g. Johnson and Hanson 1962, Folkow, Sommerschein and Wright 1971, Lundvall and Järhult 1976). AP, SAP, SVP and VP were recorded with Statham transducers.

The muscle preparation with intact circulation was placed in a specially designed hermetically sealed temperature-controlled ( $38^{\circ}\text{C}$ ) plethysmograph (Grände, Järhult and Mellander 1974) filled with Tyrode's solution. The fluid in the plethysmograph was in connection via a tubing with an external open reservoir mobile in the vertical direction to permit variation of the hydrostatic fluid pressure in the plethysmograph and hence of extravascular pressure to desired levels in relation to atmospheric pressure. By changing the height of the reservoir, all parts of the muscle vascular bed could thus be exposed to graded increases or decreases in vascular transmural pressure ( $P_T$ ) which could be applied at any desired rate  $dP_T/dt$  without interference with the arterio-venous pressure difference. This plethysmographic technique also permitted recordings of tissue volume variations.

Recordings of total and segmental vascular resistances in the muscle region. Vascular resistances were determined as the ratio: mean perfusion pressure/mean arterial blood flow ( $Q$ ) and expressed in peripheral resistance units PRU ( $\text{mm Hg ml}^{-1} \text{ min}^{-1} 100 \text{ g tissue}^{-1}$ ). The following definitions were used: total vascular resistance ( $R_{\text{tot}}$ )  $(AP - VP)/Q$ ; proximal arterial resistance ( $R_{\text{prox}}$ )  $(AP - SAP)/Q$ ; microvascular resistance ( $R_{\text{micro}}$ )  $(SAP - SVP)/Q$ ; and large vein resistance ( $R_{\text{ven}}$ )  $(SVP - VP)/Q$ . In most cases continuous recordings of the above vascular resistances were obtained with the aid of resistance meters (see next chapter). In some cases resistance was calculated manually. All vascular parameters were recorded on a ten channel Grass Polygraph.

The distinction between proximal arterial vessels, microvessels and large veins refers to the site from which SAP and SVP were monitored. The used method to measure these pressures is indirect and the recordings represent microvascular pressures as transmitted via communicant vessels from the microcirculation to the distal ramifications of the sural artery and vein. Arguments were given (papers II and VI) for the validity of this approach to measure microvascular pressures in the studied muscle region.

Especially useful for the interpretation were some recent vital microscopy observations of the size of the parent arterial vessels at the site from which

the most proximal communications emanate (20 - 25  $\mu\text{m}$  i d ; e g Eriksson and Myr-hage 1972) and further some data on simultaneous vascular diameter and micro-pipette pressure measurements along the vascular tree in skeletal muscle (e g Fronck and Zweifach 1975) It can be concluded that SAP in all the present in-vestigations was monitored from arterial microvessels with an approximate ave-rage inner diameter of about 25  $\mu\text{m}$  implying that the so called 'proximal arte-rial vessels' were larger than and the 'arterial microvessels' smaller than this dimension (the size of 15 - 20  $\mu\text{m}$  stated in paper II and III seems to be an underestimation)

The site from which SVP was recorded is more difficult to precisely define due to the quite flat pressure drop curve across the venous microvessels (Fronck and Zweifach 1975) Approximate deduction suggests that SVP was monitored from venules of about 20 - 40  $\mu\text{m}$  (i d ) Despite the fact that the capillaries and the minute veins were included in the microvascular resistance recordings the myogenic reactions to be described in all probability occurred mainly on the precapillary side as discussed for instance in paper II

Correct segmental resistance measurements presuppose that arterial blood flow is uniform throughout the vascular bed It was concluded (paper II) that this demand was reasonably well fulfilled under most experimental conditions in th present investigations with the possible exception for the large vein section during phasic change of a vascular transmural pressure when large vein re-sistance recordings might be somewhat unreliable

From these and other methodological considerations presented in the investiga-tions it may be concluded in summary that the experimental approach used can be considered to provide useful and reliable observations of myogenic reactivity to transmural pressure stimuli in the whole vascular bed of skeletal muscle as well as in its segments the proximal arterial vessels and the microves-sels and with some reservation also the large veins

# DESIGN OF AN ELECTRONIC DIFFERENTIAL PRESSURE FLOWMETER AND A RESISTANCE METER FOR CONTINUOUS MEASUREMENT OF VASCULAR RESISTANCE (paper I)

The aim of the present investigations was to study dynamic and static myogenic reactivity to phasic and steady-state changes of vascular transmural pressure in the vascular bed of the lower leg muscles in the cat, and to differentiate between the responses in proximal arterial vessels, microvessels and large veins. Such studies require specialized technical instrumentation which permits accurate and continuous recordings of blood flow in a range as small as 1 ml/min and further reliable continuous and simultaneous recordings of segmental vascular resistance responses even during short lasting phasic shifts of vascular transmural pressure. Most conventional flowmeters for continuous measurement like the electromagnetic one show poor technical characteristics in this low flow range. A special flowmeter and a vascular resistance meter were therefore designed which satisfied the above-mentioned demands.

The flowmeter was based on the differential pressure principle. This principle has been applied in several previous flowmeter designs (e.g. De Burgh Daly 1926, Lawson and Holt 1939, Ueno and Takenaka 1955) but has been of little practical use in biological research because of several inherent problems. Thus the very small pressure fall over the flowmeter created by flow has been difficult to record exactly with the so far used mechanical, optical or strain gauge manometers. The previous instruments have also suffered from nonlinearity and considerable problems related to the phase lag of the arterial pulse pressure etc.

Recent developments in the field of piezo-resistive pressure transducers, especially the hybrid IC type (National Semiconductor Corp.) offer the possibility of precise and reliable measurements of minute differential pressures. The present flowmeter in which such a transducer was used and in which the problems with the pulse pressure phase lag were largely overcome, was shown to provide a means for reliable and linear recordings of mean (or pulsatile) blood flow even in the small flow range encountered in the studied cat skeletal muscle region.

Fig. 1 illustrates the flowmeter principle (left part) in combination with a resistance meter (right part, see below) as adopted for the present measurements of total and segmental vascular resistances in the skeletal muscle region.

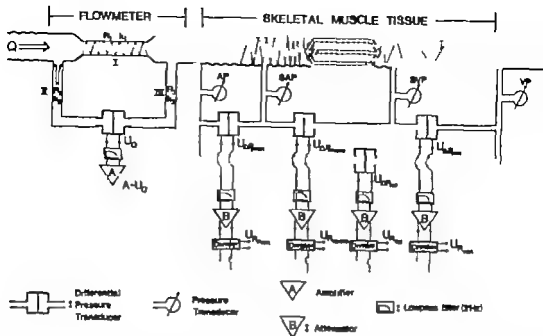


Fig 1 Schematic illustration of the differential pressure flowmeter (left part) and the resistance meters (right part) as adopted for the present studies on the vascular bed of skeletal muscle. The flowmeter consists of tubes I, II and III where  $R$  denotes tube resistance,  $k$  the tube elasticity modulus and  $U_Q$  the flowmeter signal. The resistance meters consist of differential pressure transducers giving pressure signals  $U_{AP}$  and of electronic divider circuits providing continuous recordings of total resistance ( $U_{R_{TOT}}$ ), proximal arterial resistance ( $U_{R_{prox}}$ ), microvascular resistance ( $U_{R_{micro}}$ ) and large vein resistance ( $U_{R_{ven}}$ ).

The flowmeter consists of a main tube (I) across which blood flow is diverted from the proximal to the distal end of the severed artery. The pressure drop over this tube is measured with a differential pressure transducer with virtually no membrane displacement via tubes II and III. It was highly essential that pulsatile pressure was transmitted to either side of the differential pressure transducer without any significant phase lag. The latter was accomplished by choosing appropriate values for the tube resistances  $R_1$ ,  $R_2$  and  $R_3$  as well as for their elasticity moduli  $k_1$ ,  $k_2$  and  $k_3$  (Fig 1). It was calculated that no phase lag at zero flow would exist if the relation  $R_2/k_2 = (R_3 + R_1)/k_3$  was satisfied. This was also experimentally confirmed by the absence of arterial pulse pressure oscillations during occlusion of the artery just distal to the entrance of tube III. Further tests (unpublished) have demonstrated that accurate mean flow recordings were achieved with this flowmeter for flows in the range from zero up to 20 ml/min for pulsation frequencies up to 4 Hz at normal pulse pressure amplitude.

Since blood flow is diverted extracorporeally on the arterial side in tube 1 of the flowmeter the tube was made of an inert silicone rubber material moulded in such a way that the inner surface of the tubing was entirely smooth. Test experiments demonstrated that this flowmeter caused no hemolysis nor any release of vasoactive substances: the latter evidenced by virtually unchanged vascular resistance in the steady-state after insertion of the flowmeter circuit.

This flowmeter was used in all the present investigations and was found to be linear in and well above the flow range encountered in these studies. Baseline drift was small.

The vascular resistance meters (see Fig. 1 right part) consisted of electronic divider circuits into which were fed the signals from differential pressure transducers which recorded the pressure drop across the whole vascular bed and its segments and the signal from the arterial flow meter.



### Static and dynamic myogenic reactivity in the vascular bed of skeletal muscle (papers II and III)

In most previous investigations *in vivo* myogenic reactions have been studied in response to change of arterial and/or venous pressure. The interpretation of myogenic responses to such changes of intravascular pressure is complicated by the fact that induced passive alterations of blood flow lead to simultaneous interference with the metabolic vascular control system. With the present approach in which all parts of the vascular bed were exposed to change of vascular transmural pressure ( $P_T$ ) by variations of extravascular (plethysmographic) pressure such flow induced influences of the metabolic control system were largely avoided since the arterio-venous pressure difference was maintained constant.

To reveal a possible dynamic or rate-sensitive component in the myogenic control besides the previously described static component vascular resistance changes in the sympathectomized skeletal muscle were studied in response to a given increase or decrease in vascular transmural pressure  $P_T$  applied at different rates  $dP_T/dt$ . The resistance responses were thus followed both during the dynamic phase of the transmural pressure change and in the steady-state (static) phase of constant increased or decreased pressure.

In a first series of expts (paper II) such responses were studied in the whole vascular bed as well as in its segments: the proximal arterial vessels, the microvessels, and the large veins (for definitions see Methods). The expts were performed on the normal myogenically reactive as well as on the completely paralysed (papaverine-dilated) vascular bed. Vascular transmural pressure in this study was always changed by a total of 40 mm Hg, but the pressure change was applied at two distinctly different rates: viz. in 15 s and 120 s corresponding to  $dP_T/dt$  values of 2.7 and 0.33 mm Hg/s (positive and negative).

The results from these expts are summarized in Fig. 2. The paralysed vascular bed (dotted curves) showed as expected an entirely passive behaviour in terms of decreased resistance in all vascular sections during the whole period of increased transmural pressure. In the vascular bed with preserved basal tone

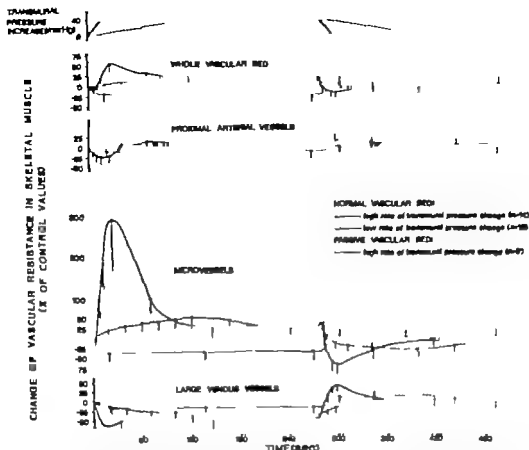


Fig. 2 Compiled data for total and segmental vascular resistance changes in the normal myogenically reactive and in the passive myogenically inactive sympathectomized vascular bed of skeletal muscle evoked in response to increased and subsequently decreased vascular transmural pressure ( $\pm 20$  mm Hg) applied at high (2.7 mm Hg/s) and low (0.33 mm Hg/s) rates. Note the presence of both a rate-dependent dynamic and bi-directional component and a static component in the myogenic microvascular response and the passive behaviour in the paralysed vascular bed.

(solid and dashed curves where the former show the responses to the high and the latter to the low rate of the transmural pressure change) a passive distension effect of the transmural pressure rise was maintained only in the large vein section but was very transient in the others. Thus within few seconds after the beginning of the pressure increase resistance rose to exceed the control value in the whole vascular bed and in the microvessels and with some longer delay also in the proximal arterial vessels demonstrating active constrictor responses in these sections to the transmural pressure stimulus. In the microvascular section the constrictor responses reached high peak values shortly after completion of the transmural pressure rise and these effects were also reflected in overall regional vascular resistance. The magnitude of these peak constrictor responses were however much greater with the high (2.7 mm Hg/s) than the low (0.33 mm Hg/s) rate of the transmural pressure increase.

The later steady state constrictions in response to constant increased transmural pressure on the other hand were small and of equal size at both rates. When transmural pressure was decreased back to the control level at the same specified rates ( $dP_T/dt$  2.7 and 0.33 mm Hg/s) the pattern of response was reversed with pronounced active microvascular dilation far below the control vascular tone in the period of phasic decline of pressure. These responses were also clearly graded in relation to the rate of the pressure decline. Vascular tone then gradually returned to the initial control level. These microvascular dilator responses were also reflected in overall vascular resistance. The described pattern of vascular response was entirely unaffected by adrenergic blockade (phenoxybenzamine and propranolol) strongly indicating that the reactions were of local myogenic origin.

These results demonstrate the existence of a clearcut two-component effector response in the microvessels to altered vascular transmural pressure. The initial component developing during the period of changing pressure was much larger when evoked by high than low rate of transmural pressure change strongly indicating that the response was rate-dependent. The subsequent steady state component revealed during the period of constant increased pressure was small and rate independent. This microvascular response pattern after exclusion of some alternative explanations (see paper II) was interpreted to demonstrate the existence of a dynamic rate-sensitive as well as a static component in the myogenic response of the microvessels to changed transmural pressure ( $dP_T/dt$  and  $P_T$  respectively) in skeletal muscle. This conclusion was strongly supported by the close resemblance between this pattern of microvascular response and the analogous pattern response of the rat portal vein to passive static stretch and shortening applied at various rates the latter study providing unequivocal evidence for dynamic and static myogenic vascular reactivity (see Introduction).

The rate-dependent microvascular constrictor response at the  $dP_T/dt$  value of 2.7 mm Hg/s (Fig. 2) was 10-15 times greater than the static steady-state response. It was concluded that the microvessels in muscle exhibit strong myogenic reactivity to transmural pressure stimuli though the behaviour was dominated by the dynamic rather than the static characteristics of the stimulus. The proximal arterial vessels on the other hand seemed to exhibit only static myogenic reactivity to a transmural pressure change (Fig. 2) whereas the large vein section which responded passively appeared to lack myogenic reactivity entirely.

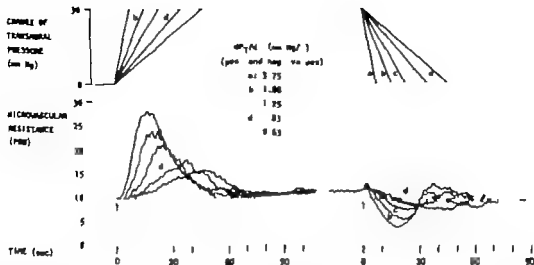


Fig 3 Dynamic and static myogenic microvascular resistance responses in sympathectomized skeletal muscle evoked by increase and a subsequent decrease in vascular transmural pressure ( $P_T$ ) by 30 mm Hg applied at 5 different rates  $dP_T/dt$  (see key)

More detailed information about the stimulus-effector characteristics of the dynamic and static components in myogenic microvascular control was obtained from another series of expts (paper III). The analysis was here performed during graded changes of static vascular transmural pressure ( $P_T$ ) from 0 to 50 mm Hg applied at different rates  $dP_T/dt$  in the entire range from +7.5 to -7.5 mm Hg/s.

Fig 3 illustrates in a representative expt how microvascular resistance in skeletal muscle was influenced by a given total increase in  $P_T$  of 30 mm Hg applied at five different rates in the  $dP_T/dt$  range from 0.63 to 3.75 mm Hg/s and by subsequent decrease in  $P_T$  back to the control level at the same given (negative) rates. To permit direct comparison the microvascular resistance responses to different  $dP_T/dt$  stimuli have been displayed on top of each other in this figure. Change of  $P_T$  led to the following general pattern of microvascular resistance response. Few seconds after the onset of the  $P_T$  rise resistance started to increase and reached a peak or transient plateau shortly after  $P_T$  had attained the final constant level of 30 mm Hg above the control value. Resistance then declined again despite the fact that static transmural pressure was maintained at the high constant level and it stabilized at a steady-state level only a few resistance units above the initial control value. When  $P_T$  was decreased again the microvessels showed clearcut active dilator responses graded in relation to the rates of the pressure decline. The subsequent recovery of microvascular tone was characterized by an overshoot or an oscillatory

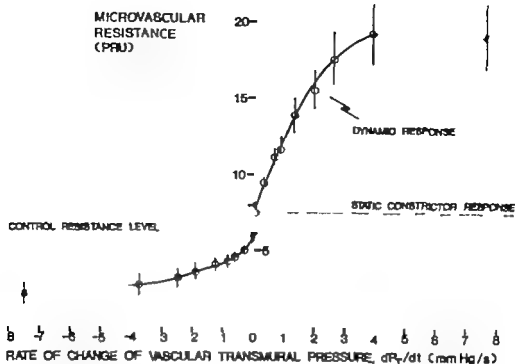


Fig. 4 Diagram illustrating the average dynamic myogenic microvascular constrictor and dilator responses in skeletal muscle to increased and subsequent decreased vascular transmural pressure by 30 mm Hg applied at various rates from +7.5 mm Hg/s to -7.5 mm Hg/s ( $n = 7$ ). The average static constrictor response to constant increased  $P_T$  by 30 mm Hg above the control level is also shown.

behaviour before resistance stabilized at its initial control value.

These results further strengthen the view of a rate-sensitive dynamic and a static regulatory mechanism in myogenic microvascular control and show in greater detail that the rate-sensitive mechanism in operation during the phase of the transmural pressure change is bi-directional causing graded excitatory constrictor effects in response to positive and graded inhibitory dilator effects in response to negative values of  $dP_T/dt$ . Note that the static myogenic response revealed during the phase of steady-state constant increased transmural pressure was quite small compared to the dynamic responses.

Fig. 4 summarizes the results for the average dynamic microvascular constrictor and dilator responses to  $dP_T/dt$  stimuli over the range from +7.5 to -7.5 mm Hg/s. The average static constrictor response to the standardized increase in  $P_T$  by 30 mm Hg is also illustrated. It can be seen that the microvessels exhibited smoothly graded dynamic constrictor responses to excitatory  $dP_T/dt$  stimuli (pressure increase) in the lower range of test with a maximum reached at a  $dP_T/dt$  value of about +4 mm Hg/s. At higher rates the resistance curve

levelled off demonstrating that extreme myogenic effector reactions are not elicited. Similarly there were graded active microvascular dilator responses to inhibitory  $dP_T/dt$  stimuli (pressure decrease) maximal dilator effects being reached at the  $dP_T/dt$  value of  $-4$  to  $-5$  mm Hg/s. Further analysis indicated that the resistance change per unit time  $dR_{micro}/dt$  was also graded in relation to  $dP_T/dt$  both for positive and negative values.

The static myogenic microvascular responses were shown to be graded in relation to the amplitude of the  $P_T$  increase within the range up to about 40 mm Hg above normal transmural pressure. The static transmural pressure stimulus when maximal increased microvascular resistance by only about 3 PRU above control which should be compared with the effect of a strong dynamic transmural pressure stimulus which was 10-15 times more effective in exciting the smooth muscle of the microvessels.

The size of the dynamic microvascular constrictor response though basically dependent on the magnitude of the dynamic stimulus  $dP_T/dt$  also seemed related to the duration of the latter and/or the amplitude of the static transmural pressure change (paper III). This quite complex effector response was tentatively explained by gradual recruitment of an increasing number of dynamic receptor units with different thresholds as  $P_T$  rises. A recent mathematical analysis of myogenic microvascular reactivity (Borgström and Grände 1979) suggested that this phenomenon alternatively or in addition could be ascribed to a increase in gain implicit in the inverse fourth power relationship between vascular resistance and vessel radius according to the law of Poiseuille.

The approach used in the present investigation by which myogenic reactivity was studied during equal increase of transmural pressure in all sections of the vascular bed permits basic description of the characteristics of its static and dynamic components: the latter in terms of the transient effector response to a short lasting phasic transmural pressure change. This experimental approach resembles the hemodynamic in vivo situation in dependent vascular beds when exposed to hydrostatic load as created for example upon shift from the supine to the erect body posture. The initial load on the dependent vascular bed caused by kinetic energy then exceeds several times the later steady-state hydrostatic load and the very marked microvascular resistance increase noted in the present expts (Fig. 2 and 3) might then be needed to protect the vessels against damage. It is quite likely however that the described rate-sensitivity plays a more universal role in myogenic vascular control with biological

implications analogous for instance to those in the muscle spindle of skeletal muscle with its highly differentiated dynamic and static receptor organization (e.g. Matthews 1964). The rate-dependent regulatory mechanism in myogenic circulatory control might serve the important function to increase its rapidity, stability and sensitivity like in many other servo control systems (see General Discussion).

### Role of intravascular static and dynamic transmural pressure stimuli in the development of normal basal vascular tone (paper IV)

The previous section was concerned with myogenic vascular responses to experimentally applied changes in vascular transmural pressure. In the expts. to be described in this section an attempt was made to determine to what extent normal intravascular pressure on the arterial side of the muscle vascular bed via static and dynamic transmural pressure stimuli might be responsible for the initiation and maintenance of normal myogenic basal vascular tone. For this purpose the characteristics of the intrinsic basal vascular tone in the adrenergically blocked skeletal muscle were analysed with regard to its extent and site along the vascular bed, its dependence on static mean arterial blood pressure, distension and dynamic pulse pressure distension, and finally its sensitivity to local metabolic influence. The expts. were designed in such a way that by analogies to electrophysiological studies of myogenic reactivity in other vascular smooth muscle *in vitro*, some tentative considerations could be given about the cellular events behind basal vascular tone.

The study first confirmed previous observations that basal vascular tone in skeletal muscle, which apparently is of myogenic nature (e.g. Folkow 1962), was pronounced in proximal arterial vessels and in microvessels but low in large veins. As a first approach to the problem of its underlying mechanisms a series of expts. was performed to reveal the extent to which normal basal vascular tone on the arterial side can be decreased by a maximal vascular transmural pressure stimulus of inhibitory nature. For this purpose the vascular bed at normal intravascular distending pressure was exposed to a strong negative  $dP_T/dt$  stimulus, known from the data in the previous section to cause effective vasodilation.

The results demonstrated that the applied transmural pressure stimulus ( $P_T$  decline of 40 mm Hg applied at the rate of -5 mm Hg/s) was capable to very effectively inhibit basal tone in the microvessels where resistance decreased by almost 90 % of that evoked by papaverine the latter substance known to entirely abolish vascular tone whatever its cause. Such an inhibitory stimulus however was much less effective in the proximal arterial vessels the basal tone (PRU) of which could be decreased by no more than about 10 % of the papaverine effect. Evidently basal tone in the microvessels is much more sensitive to the mechanism which mediating the response to an inhibitory transmural pressure stimulus than that of the more proximal vessels suggesting that basal tone in these consecutive vessels is of somewhat different nature.

In a second series of expts. the importance of normal arterial blood pressure pulsations on basal vascular tone was investigated. For this purpose, total and segmental vascular resistances in the adrenergically blocked muscle region were analysed at normal basal tone upon shift from pulsatile to non-pulsatile perfusion under maintenance of a normal and constant mean arterio-venous pressure difference. The study demonstrated, that such elimination of the arterial pulse pressure oscillations caused a pronounced inhibition of basal tone in terms of a decrease by almost 20 % in total vascular resistance in the muscle confirming previous results of Mellander and Arvidsson (1974) and Lalone (1975). The major part of this dilator response however was shown to be localized to the microvessels the resistance of which decreased by almost 35 % below control while resistance in the more proximal arterial vessels decreased by only some 10 %. It was concluded, that the normal arterial pulse pressure oscillations seem to contribute significantly to the development of basal tone though mainly in the microvessels an effect attributed to repetitive positive dynamic transmural pressure stimuli on the vascular smooth muscle evoked during the rising phase of the pulse pressure curve.

In a third series of expts. an attempt was made to reveal to what extent arterial blood pressure via its static mean pressure distension effect might contribute to the initiation and maintenance of basal vascular tone. For this purpose changes of basal vascular tone in the 'proximal arterial vessels' and the microvessels were analysed in response to decreases in arterial blood pressure and further in response to superimposed restoration of mean distending pressure to normal under maintained arterial hypotension. This restoration of vascular transmural pressure was performed by decrease of extravascular pressure. Special tests were performed to reveal possible simultaneous influence by the induced arterial hypotension. It was concluded from



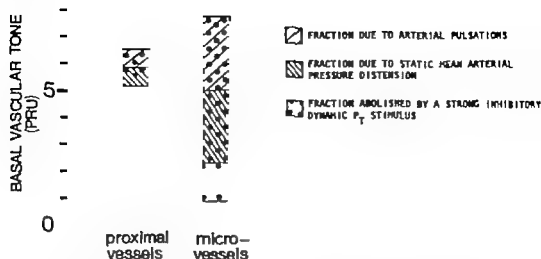


Fig 5 Histogram summarizing in approximate figures the extent of normal basal tone in proximal arterial vessels ( $>25 \mu\text{m}$ ) and microvessels ( $<25 \mu\text{m}$ ) in skeletal muscle (total height of columns) and its dependence on transmural pressure stimuli (see key). Note that a considerable fraction of the normal basal tone in the microvessels is dependent on arterial pressure both via its static distension effect related to mean arterial pressure and its dynamic pulse pressure distension. Note further that a strong inhibitory dynamic transmural pressure ( $P_T$ ) stimulus ( $dP_T/dt = -5 \text{ mm Hg/s}$ ) could virtually abolish basal tone in these vessels. Basal tone in the proximal arterial vessels was much less affected by such transmural pressure stimuli.

different expts that normal arterial blood pressure via its static mean pressure distending effect significantly contributes to the development of basal tone on the arterial side though more markedly in the microvessels than the proximal arterial vessels. In rough figures static mean arterial pressure distension seemed to be responsible for some 35 % of the normal basal tone in the former but only for some 10 % in the latter.

The results of these investigations can be summarized schematically and in rough figures as illustrated in Fig 5. The extent of smooth muscle tone on the arterial side in the proximal arterial vessels ( $>\text{about } 25 \mu\text{m i d}$ ) and the microvessels ( $<\text{about } 25 \mu\text{m}$ ) is here expressed in terms of its active vascular resistance corollary (PRU) i.e. the resistance at the normal level of basal tone minus the small physical resistance remaining after complete smooth muscle relaxation induced by papaverine. Under control conditions basal tone corresponded to an average of 6.6 PRU in the proximal arterial vessels and 7.8 PRU in the microvessels. The normal arterial blood pressure thus seemed to be partially responsible for the initiation and maintenance of this basal tone both via its pulse pressure oscillations and its mean pressure level the former apparently creating a repetitive dynamic and the latter a static trans

transmural pressure stimulation of the smooth muscle. These two stimuli were about equally effective. The figure might illustrate more directly that the fraction of basal tone which could be attributed to these two stimuli was much greater in the microvessels than in the proximal arterial vessels. Experimental application of a seemingly maximal inhibitory dynamic transmural pressure stimulus ( $dP_T/dt = 5 \text{ mm Hg/s}$ ) caused a very effective still not complete inhibition of the normal basal tone in the microvessels but such a stimulus had only a slight effect on tone in the more proximal arterial vessels.

The results of this study which in different ways demonstrate that basal tone is much more dependent upon and sensitive to transmural pressure stimuli in the microvessels than in the proximal arterial vessels strongly suggest that basal tone is of somewhat different nature in these two vascular sections. The functional behaviour reported for the microvessels (Fig 5) might in fact refer to the entire microcirculation to which the smallest ramification of the proximal arterial vessels most likely belong in view of the somewhat arbitrary delineation between proximal arterial vessels and microvessels with the present approach viz at the vessel dimension as small as about  $25 \mu\text{m}$ .

Even if basal tone in both the arterial microvessels and the more proximal arterial vessels in skeletal muscle by conventional definition can be considered to be of myogenic origin (e.g. Folkow 1962) the described functional discrepancies between the two sections (Fig 5) might suggest somewhat different cellular mechanisms behind their basal vascular tone. Circumstantial evidence will be presented in the General Discussion section for the tentative view that vascular smooth muscle in the arterial microvessels is mainly of the spike-generating type and that in the somewhat larger arterial vessels more of the non spike-generating contractile type.

#### Adrenergic influence on myogenic microvascular reactivity in skeletal muscle (papers V and VI)

The concept of myogenic vascular control has been much debated since it was originally proposed by Bayliss (see Introduction). One of the reasons has been that even seemingly well designed expts can exhibit poor or occasionally failing myogenic reactivity. Such experiences must not necessarily be taken as evidence against the myogenic hypothesis which occasionally seems to have been the case but might merely imply that the myogenic vascular control system by some mechanism is vulnerable to experimental intervention.

This problem was approached in paper V which revealed that stress induced release of catecholamines from adrenal medulla via its  $\beta$  adrenergic influence could explain much of the decreased myogenic reactivity under experimental conditions. The evidence was as follows. The sympathectomized vascular bed of skeletal muscle exhibited impaired dynamic myogenic reactivity and decreased microvascular tone in the early period after completion of surgery but gradual recovery towards normal within about an hour of subsequent rest. These findings indicated the presence of some inhibitory factor(s) which gradually disappeared during extended rest and therefore most likely was activated during the surgical and other experimental intervention. Early post-surgical  $\beta$ -adrenergic blockade with propranolol caused almost immediate restoration of myogenic reactivity and of vascular tone towards normal indicating that the inhibitory effect to a great extent was evoked by  $\beta$  adrenoceptor stimulation of circulating catecholamines. Poor myogenic reactivity reported in some previous investigations on more or less traumatized preparations might thus simply be explained by stress induced  $\beta$  adrenergic inhibitory interaction with the myogenic control system.

The interaction between adrenergic and myogenic regulatory mechanisms was analysed more systematically in paper VI in an attempt to reveal in particular whether the  $\beta$  adrenergic vascular control system might serve the function to modify myogenic reactivity also under normal physiological conditions. For this purpose myogenic vascular reactivity to a standardized transmural pressure stimulus ( $P_T$  increase of 40 mm Hg applied at a constant rate of 2.5 mm Hg/s) was studied in skeletal muscle before and during graded neural and humoral adrenergic influence on the vascular bed within the physiological range.

Fig. II shows effects of graded sympathetic nerve stimulation (0–12 Hz) on microvascular resistance (abscissa) and on the superimposed dynamic myogenic constrictor response (ordinate) to the standardized test transmural pressure stimulus on the muscle vascular bed with intact  $\alpha$  and  $\beta$  adrenoceptors (lower curve) and after effective  $\beta$  adrenergic blockade (upper curve). Sympathetic nerve stimulation caused as expected an increase in microvascular tone (abscissa) graded in relation to the excitation rate both on the intact and the  $\beta$ -blocked vascular bed. This constrictor response was somewhat less pronounced in the former especially at high stimulation rates indicating an inhibitory  $\beta$  adrenergic microvascular resistance effect of neural excitation in the intact vascular bed which confirms previous results by Lundvall and Jörhult (1976). The superimposed myogenic microvascular constrictor response to the

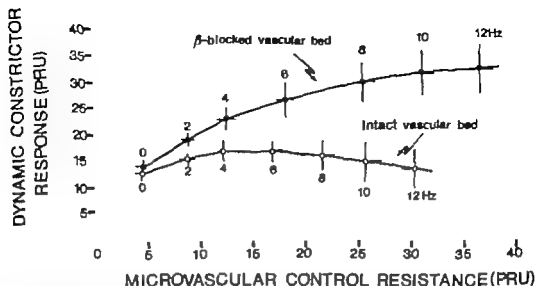


Fig 5 Effects of graded sympathetic nerve stimulation on microvascular resistance (abscissa) and on the superimposed dynamic myogenic constrictor response (ordinate) to a standardized test transmural pressure stimulus on the vascular bed with intact adrenoceptors (lower curve) and after effective  $\beta$ -adrenergic blockade (upper curve)

test stimulus (ordinate) was of almost the same magnitude at all rates of sympathetic stimulation in the intact vascular bed but significantly increased when the  $\beta$ -adrenoceptors were blocked. Since the neural  $\alpha$ -adrenergic influence can be considered equal in the two series of experiments, the depicted significantly smaller dynamic constrictor response in the intact vascular bed must be ascribed to a neural  $\beta$ -adrenergic inhibitory influence on myogenic reactivity. It was concluded that sympathetic nerve activation via a  $\beta$ -adrenergic component exerted an effective inhibitory action on dynamic myogenic microvascular reactivity graded in relation to the nerve excitation rate. Such graded  $\beta$ -adrenergic inhibitory effects were also revealed during infusions of adrenaline and noradrenaline where adrenaline was shown to be the more effective inhibitor and capable to decrease the myogenic response well below the control level.

The quite remarkable effect demonstrated in Fig. 6 in terms of gradually increasing dynamic myogenic constrictor responses in the  $\beta$ -blocked vascular bed during sympathetic nerve stimulation (and also during catecholamine infusion) did not seem to be explained by reinforcement of myogenic reactivity by  $\alpha$ -adrenoceptor stimulation. As will be discussed, it more likely can be ascribed to a physical effect of the concomitantly raised microvascular tone leading to an increase in gain implicit in the inverse fourth power relationship between microvascular resistance and internal vessel radius according to Poiseuille's

law Since this reinforcing phenomenon was not observed on the vascular bed with intact adrenoceptors (see Fig 6) It was suggested that the  $\beta$  adrenergic mechanism in neural (and humoral noradrenergic) sympathetic control serves the seemingly important function to compensate for such a physical effect, implying the development of an almost equally large myogenic resistance response to a given transmural pressure stimulus irrespective of the prevailing level of vascular tone (see General Discussion)

The described even more pronounced inhibitory effects of adrenaline might be encountered under in vivo conditions only during relatively severe stress and could then serve the function to facilitate capillary exchange by decrease of myogenic tone and reactivity in resistance vessels and precapillary sphincters (cf Lundvall and Hillman 1978)

## GENERAL DISCUSSION AND SUMMARY

The studies summarized in this paper deal with the active smooth muscle responses of the vascular bed of skeletal muscle to the mechanical force exerted by intravascular or transmural pressure. Such locally mediated changes of vascular tone have by convention been denoted myogenic reactivity. The experimental approach was such as to permit observations of myogenic responses without significant simultaneous interference with the local metabolic control system. This was accomplished by exposing the whole vascular bed under study to changes of vascular transmural pressure via variations in extravascular pressure thus avoiding alterations of the perfusion pressure and hence passive changes in blood flow. With this technique the vascular bed could be exposed to graded change in vascular transmural pressure ( $P_T$ ) both with regard to its amplitude (static transmural pressure stimulus) and its rate  $dP_T/dt$  (dynamic transmural pressure stimulus). The experimental design allowed simultaneous and continuous recordings of vascular resistance in the whole vascular bed as well as in its longitudinal segments: the proximal arterial vessels ( $>$ about  $25 \mu\text{m}$  i.d.) the microvessels ( $<$ about  $25 \mu\text{m}$ ) and the large veins.

The results were interpreted with regard to their most direct physiological implications in the foregoing sections. Their possible functional significance will be discussed below in a wider and therefore perhaps somewhat more speculative perspective.

The data in papers I) and III) demonstrated the existence of a dynamic or rate sensitive component in addition to the previously known static component in the myogenic vascular response to changed transmural pressure. Rate-sensitivity in myogenic control however seemed to be present only in the microvessels of skeletal muscle whereas static myogenic reactivity was present also in the proximal arterial vessels. The large veins on the other hand seemed to be devoid of myogenic reactivity. Analysis of the stimulus-effector characteristics in microvascular myogenic control revealed that the dynamic resistance responses developing during the phase of changing  $P_T$  were pronounced and distinctly graded in relation to the magnitude of the  $dP_T/dt$  stimulus both with regard to the amplitude of the resistance response and the rate of the resistance change per unit time. Moreover rate-sensitivity in myogenic microvascular control was found to be bi-directional eliciting excitatory effects (con-

striction) in response to positive and inhibitory effects (dilation) in response to negative values of  $dP_T/dt$ . Maximum dynamic constrictor responses were obtained at a  $dP_T/dt$  value of about +4 mm Hg/s and maximum dilator responses at a negative  $dP_T/dt$  value of about the same order of magnitude. With stronger dynamic transmural pressure stimuli the effector responses levelled off. The static responses revealed in the steady-state phase of constant increased  $P_T$  were comparatively small and graded in relation to the amplitude of the  $P_T$  increase. A strong dynamic transmural pressure stimulus was some 10-15 times more effective in increasing microvascular tone than a strong static stimulus.

It is evident that the dynamic component in myogenic microvascular control will serve the function to accomplish very prompt effector responses to a given transmural pressure change. There is much to indicate, however, that rate-sensitivity is of importance not only for such transient effector responses but might be of more generalized physiological significance in myogenic vascular regulation. A rate-dependent regulatory mechanism, especially when bi-directional, is of great importance in many servo-control systems in order to increase rapidly stability and sensitivity and thereby regulatory efficiency. The described biological data taken together indicate that the dynamic component in myogenic microvascular control subserves such purposes. For proper function, most servo-control systems require a smoothly graded relation between the rate-dependent input signal and the resulting output signal (Melzer and Schultz 1969). Such relations were demonstrated for the dynamic transmural pressure stimulus and its effector response, the latter in terms of microvascular resistance change as well as rate of the resistance change per unit time. This also applies to stretch-induced dynamic myogenic reactivity in the rat portal vein and its underlying change of pacemaker-generated spike discharge.

More detailed information about the quite complex variables involved in myogenic vascular control in vivo might be derived from a recent mathematical analysis of the described static and dynamic myogenic responses in the microvessels to a given change of transmural pressure applied at various rates (Borgström and Grände 1979).

This mathematical model was based on a force-equilibrium equation including passive forces related to vascular transmural pressure, elasticity and wall viscosity, and the active myogenic forces related to the mentioned static and dynamic responses to change of transmural pressure. It was assumed in the approach that the myogenic responses were related to and triggered by changes of

wall tension an opinion tentatively suggested in some previous publications (Johnson and Wayland 1967 Sigurdsson Johansson and Mellander 1977) Great resemblance was demonstrated between the microvascular resistance curves obtained with this model and the corresponding curves presented in paper III of the present study indicating that the model quite adequately can describe myogenic microvascular resistance responses to transmural pressure stimuli These results supported the myogenic hypothesis in general and in particular the concept of an important rate-sensitivity in myogenic microvascular control They strongly indicated that microvascular myogenic reactions are triggered by and related to change of wall tension i.e. to  $\sigma$  (static response) and to  $d\sigma/dt$  (dynamic response) The mathematical model predicted that  $\sigma$  increases above control during a transmural pressure increase implying a tendency of a positive feedback in myogenic control However after an initial rise  $d\sigma/dt$  declined implying an inherent negative feedback in myogenic regulation which significantly can help to stabilize this circulatory control system

A tentative description of myogenic autoregulation was presented by Mellander (1978) in which vascular tone during increased transmural pressure was suggested to oscillate around an increased level as a result of mainly repetitive dynamic transmural pressure stimuli on the vascular smooth muscle A damped oscillatory behaviour of microvascular tone was a characteristic finding after a fast change of vascular transmural pressure (paper II) or of arterial pressure (paper IV) when myogenic reactivity was well preserved Oscillatory responses are typical for vasomotion and have also been reported in some previous hemodynamic studies (e.g. Folkow 1953 Stainsby and Rankin 1961) though without convincing explanations It is known however that control systems may develop an oscillatory behaviour if a first derivative (rate-dependent) component as well as higher order derivative component(s) are present (Halza and Schultz 1969) Although not specifically analysed in the present papers a second (or higher order) derivative component is present in myogenic control due to biological inertia to time delay and to the presence of action potential afterdischarge the latter evidenced for myogenic reactivity in vitro (Johansson and Mellander 1975) Incorporation of such characteristics in future theories of myogenic regulation might lead to much-improved understanding of the mode of operation of this control system

It has sometimes been invoked that the myogenic control system could be self limiting insofar that a myogenic smooth muscle shortening in response to raised distending pressure automatically would abolish the primary receptor stimulus



If the receptors were sensitive to change in length (e.g. Gaskell and Burton 1953) Such self limitation is less likely to occur with myogenic receptors sensitive to wall tension and its rate of change  $da/dt$  being largely independent of absolute muscle length

Myogenic reactions to transmural pressure stimuli are effected by increases or decreases of the normal myogenic basal tone. In an attempt to get deeper insight into the interaction between myogenic reactions and basal tone the characteristics of the latter were investigated in some detail (paper IV). These studies revealed that normal basal tone in the microvessels seemed to be intimately dependent on the arterial blood pressure level and to a significant extent (some 70 %) initiated and maintained by the combined effects of its static mean pressure distension and its dynamic pulse pressure distension. Microvascular tone could be virtually abolished by a transmural pressure decrease applied at fast rate i.e. by a strong inhibitory dynamic transmural pressure stimulus. Basal tone in the proximal arterial vessels on the other hand was little affected by arterial pressure and almost irresponsive to inhibitory dynamic transmural pressure stimuli. Another difference was that basal tone in microvessels was much more sensitive to metabolic stimuli than that in the proximal arterial vessels. It was concluded that basal tone in the arterial microvessels and in the more proximal arterial vessels at least in part is of quite different nature.

The demonstration that normal microvascular basal tone to such a significant extent is depending upon static and dynamic transmural pressure stimuli created by the normal arterial blood pressure might offer a very simple explanation of myogenic autoregulatory phenomena in the microcirculation. Fall of arterial blood pressure within a certain range would lead to a graded inhibition of basal tone simply due to partial elimination of its initiating static and dynamic pressure stimuli. Conversely raised arterial pressure might lead to reinforced vascular tone due to increased static mean pressure distension and to the usually associated increase in the amplitude and/or frequency of the pulse pressure oscillations. The fact that basal tone in the proximal arterial vessels was largely independent of arterial blood pressure per se might then possibly explain why autoregulation of flow occurs only within a limited range of arterial pressures.

Mention should be made in this context that dynamic transmural pressure stimulation of the vascular bed might not solely be affected by pulse pressure oscillations.

lations but perhaps also by segmental pressure oscillations in the longitudinal direction created for instance by the intermittent local constrictions and dilations denoted vasomotion.

It appears that the observations made in paper IV might help to shed some light on the cellular mechanisms responsible for basal vascular tone and myogenic reactions. These mechanisms are not yet experimentally defined in the arterial microvessels specifically. However, the recent in vitro study by Johansson and Mellander (1975) mentioned in Introduction has clarified the electrophysiological events behind myogenic reactivity to mechanical stimuli in the single unit spike-generating vascular smooth muscle of the rat portal vein which seems to have several characteristics in common with arterial microvascular smooth muscle. The myogenic contractile response pattern is thus quite similar in both these types of vascular smooth muscle. Both exhibit a static as well as a dynamic component in their myogenic responses of which the latter is the much more prominent one; a maximal excitatory dynamic stimulus was thus some 15 times more effective in causing contraction in vitro and microvascular constriction in vivo than a maximal static stimulus. Furthermore, a strong inhibitory dynamic stimulus was capable of abolishing contractile activity in the portal vein and, as shown in paper IV, also could effectively inhibit basal microvascular tone in vivo. Such close resemblance between the portal vein and the arterial microvessels with regard to their myogenic contractile patterns of response and stimulus-effector characteristics makes it quite likely that the myogenic responses in both types of muscle are mediated via some common cellular mechanism.

The portal vein shows a characteristic spontaneous regular rhythmic contractile activity in the control state at low preload caused by bursts of action potentials primarily generated from a pacemaker cell (Johansson and Mellander 1975). Excitatory and inhibitory myogenic effector reactions in this spike-generating type of vascular smooth muscle were shown to be mediated via facilitation and inhibition of normal pacemaker generated spontaneous spike discharge. Important for the interpretation below was the fact that a strong inhibitory dynamic stimulus caused complete abolition of electrical and hence mechanical activity.

These considerations taken together make it quite likely that the myogenic reactions to transmural pressure stimuli in the arterial microvessels might be mediated via variations in myogenic pacemaker spike discharge as well. If so

It would imply that at least a major fraction of the basal tone in the microvessels is dependent upon action potentials viz that fraction (see Fig 5) which was abolished by the inhibitory dynamic transmural pressure stimulus ( $dP_T/dt = 5 \text{ mm Hg/s}$ ). Part of this fraction of basal tone in turn might then be due to a spontaneous pacemaker discharge (like in the portal vein) and the remainder (see Fig 5) to continuous reinforcement of spike activity initiated by the normal arterial pressure via its static and dynamic transmural pressure stimuli.

Basal tone in the proximal arterial vessels was little affected by transmural pressure stimuli and its major fraction (Fig 5 white column) might therefore be virtually independent of action potentials. Smooth muscle in these vessels might then possibly be of the same non-spike-generating type as present in large arterial vessels (for ref see Johansson 1978) where tone is considered to be caused by contracture and established by graded membrane depolarizations. The small fraction of basal tone in the microvessels which remained upon exposure to the strong inhibitory transmural pressure stimulus (Fig 5 white column) might tentatively be explained by a similar mechanism. The suggested differentiation of the smooth muscle along the arterial vascular bed in skeletal muscle should not be taken as a distinct feature: considerable overlap might well exist.

The cause of the common empirical observation that myogenic reactivity can be severely depressed under experimental conditions involving trauma was investigated in paper V. It was revealed that this phenomenon which has led to serious questioning of the myogenic hypothesis to a great extent could be explained by stress induced release of catecholamines from the adrenal medulla which via a  $\beta$ -adrenergic effect evoked longlasting inhibition of myogenic reactivity during experimental intervention.

This observation led to a more detailed study (paper VI) of the interaction between adrenergic and myogenic regulatory mechanisms in an attempt to reveal whether the  $\beta$ -adrenergic vascular control system might serve the function to modify myogenic reactivity also under normal physiological conditions. The conclusion was drawn that the sympatho-adrenal system via its neural and humoral  $\beta$ -adrenergic link exerts an attenuating action on myogenic excitatory reactions which seems to be physiologically meaningful.

The finding in this study that the dynamic myogenic microvascular resistance response to a standardized  $P_T$  stimulus was of roughly the same magnitude in the

control period as during sympathetic nerve stimulation in the vascular bed with intact adrenoceptors but significantly and gradually increased during nerve excitation after  $\beta$  adrenergic blockade (Fig. 6) deserves special attention

It has been clearly established e.g. by Myers and Honig (1969) that the magnitude of the constrictor response in the resistance vessels to a given excitatory stimulus is increased with increasing level of vascular tone prior to the stimulus. This reinforcing phenomenon must be a factor that significantly adds to the complexity in overall vascular control, implying non linearity in peripheral blood flow and resistance regulation. This effect as demonstrated in Fig. 6 on the  $\beta$  blocked vascular bed was subjected to a specific investigation with the use of the above-mentioned mathematical model (Borgström and Grände 1979; and unpublished data). It was concluded from these analyses that the reinforcing phenomenon mainly could be explained by an increase in gain implicit in the inverse fourth power relationship between microvascular resistance and internal radius according to Poiseuille's law though counteracted to some extent by the concomitant effects of a shift along the smooth muscle length tension curve and of the effect of Laplace law for thick-walled vessels.

This reinforcing effect points out some of the complexities in peripheral circulatory control where regulatory mechanisms act via influences on the circumferentially oriented smooth muscle cells in the blood vessels. As regards the myogenic control system it would seem reasonable that its primary stimulus in all likelihood the transmural pressure change should result in a net effector response (e.g. blood flow or resistance change) which is directly proportional to the size of the primary stimulus irrespective of the prevailing level of vascular tone. Yet the radius factor in Poiseuille's law per se inevitably leads to vast deviation from linearity in circulatory resistance control even if partially compensated for by negative feedback inherent in effects of Laplace law and of possible local accumulation of vasodilator metabolites as blood flow decreases. These latter effects however do not suffice to make the magnitude of the myogenic response independent of the prevailing vascular tone (see Fig. 6  $\beta$  blocked vascular bed) but only provides compensation. It seems that the  $\beta$  adrenergic mechanism in integrated sympathetic and myogenic control could serve the important function to create fine adjustment hence making the myogenic control system virtually linear. This conclusion was derived from the observation on the vascular bed with intact adrenoceptors (Fig. 6 lower curve) of an almost equally large dynamic myogenic resistance response to a given primary transmural pressure stimulus irrespective of the prevailing level of adrenergic vascular tone.

## SUMMARY

This study describes in some detail the characteristics of the myogenic control system and of myogenic basal tone in the vascular bed of cat skeletal muscle as well as a pattern of interaction between myogenic and adrenergic mechanisms in circulatory control

Myogenic reactivity was studied in response to graded change of vascular transmural pressure ( $P_T$ ) both with regard to its amplitude (static stimulus) and its rate  $dP_T/dt$  (dynamic stimulus). With the aid of a specially designed pressure gradient flowmeter and a vascular resistance meter continuous recordings of vascular resistance in the whole vascular bed and in its longitudinal segments the proximal arterial vessels ( $>$ about  $25 \mu\text{m}$  i.d.) the microvessels ( $<$ about  $25 \mu\text{m}$ ) and the large veins were obtained. The following main conclusions were drawn

- 1 Myogenic vascular control in skeletal muscle is characterized by a prominent dynamic or rate-sensitive component developing during phasic change of  $P_T$  in addition to the previously known static component developing during constant increased  $P_T$
- 2 Rate-sensitivity in myogenic control was present only in the microvessels and was bi-directional causing graded excitatory effects (constriction) in response to positive and graded inhibitory effects (dilation) in response to negative values of  $dP_T/dt$ . Maximum responses were evoked at positive and negative  $dP_T/dt$  values of about  $4 \text{ mm Hg/s}$
- 3 Static myogenic reactivity was present in the proximal arterial vessels and the microvessels whereas the large veins seemed to lack myogenic reactivity entirely
- 4 A maximal dynamic  $P_T$  stimulus was 10 to 15 times more effective in increasing microvascular resistance than a maximal static stimulus
- 5 Basal myogenic tone was pronounced in the proximal arterial vessels and the microvessels but almost negligible in the large veins

- 6 The Initiation and maintenance of basal myogenic tone in the microvessels seemed to be intimately related to the arterial blood pressure both via its static mean pressure distension effect and its dynamic pulse pressure distension effect. Some 70 % of the normal microvascular tone could be attributed to these stimuli both being about equally effective.
- 7 Basal tone in the microvessels could be almost completely abolished by a maximal inhibitory dynamic  $P_T$  stimulus and it was very sensitive to metabolic stimuli. Basal tone in the proximal arterial vessels was virtually irresponsive to dynamic transmural pressure stimuli and much less sensitive to local metabolic influence.
- 8 The data indicate that basal myogenic tone in microvessels and proximal arterial vessels is of somewhat different nature. Smooth muscle in the former is tentatively suggested to be mainly of the spike-generating type. In the latter it might possibly be of the non spike-generating type.
- 9 In integrated myogenic and adrenergic vascular control myogenic reactivity seemed to be attenuated by the neural and humoral  $\beta$ -adrenergic mechanism in a manner which appeared to be physiologically meaningful.
- 10 Falling myogenic reactivity commonly observed during rough experimental intervention could to a significant extent be explained by a  $\beta$  adrenergic inhibitory effect of stress induced catecholamine release.

## ACKNOWLEDGEMENTS

For all kindness and support I have received from everyone in the house I am sincerely grateful. I especially want to express my gratitude to

Stefan Hellander my teacher and fosterer in the art of physiology who introduced me into this subject. His extensive knowledge and his logical and critical way of reasoning has exerted vital influence on me, I am grateful to his generous help and support which to a great extent contributed to the outcome of this work.

Pelle Borgström my colleague for pleasant and fruitful collaboration;

Tora Jinde Anita Ekdahl and Charlotte Brange for their patient and perfect technical assistance;

Ann Ekvall for her first-rate dynamic typing of the manuscript

Börje Johansson for stimulating discussions and constructive criticism;

Per Hagander for valuable theoretical technical discussions;

The staff of the mechanical workshop headed by Leif Nilsson in particular to Lars Åke Clementz for fruitful collaboration and personal engagement in designing the technical equipment

Olle Hamner for skilled photo technical assistance and profitable collaboration;

The staff of animal quarter at the department headed by Svein Andersen for all kind help and good service

This work was supported by grants from the Swedish Medical Research Council (B79 04X 2210 13B) and from the Faculty of Medicine Lund

## REFERENCES

- AMREP G 1912 On local vascular reactions and their Interpretation *J Physiol (Lond)* 45:318-327
- BAEZ S LAIDLAW Z & ORKIN L R 1974 Localization and measurement of microvascular and microcirculatory responses to venous pressure elevation in the rat *Blood Vessels* 11:260-276
- BASAR E & WEISS C 1969 Rate sensitivity of the mechanism of pressure induced change of vascular resistance *Kybernetik* 5:241-247
- BAYLISS W H 1902 On the local reactions of the arterial wall to changes in internal pressure *J Physiol (Lond)* 28:220-231
- BERNE R M 1964 Metabolic regulation of blood flow *Circulat Res* 15 Suppl 1:261-268
- BOHR D F 1965 Individualities among vascular smooth muscles. In: Bajusz E (ed) *Electrolytes and Cardiovascular Diseases* 342-355 S Karger Basel/New York
- BORGSTROM P & GRUNDE P-O 1979 Myogenic microvascular responses to change of transmural pressure. A mathematical approach *Acta Physiol Scand* 1979:106
- DE BURGH DALY I 1926 Blood velocity recorder *J Physiol (Lond)* 61:21
- DUHLING B R 1972 Microvascular responses to changes in oxygen tension *Circulat Res* 31:481-489
- ERIKSSON E & MYRHAGE R 1972 Microvascular dimensions and blood flow in skeletal muscle *Acta Physiol Scand* 86:211-222
- FOLKOW B 1949 Intravascular pressure as a factor regulating the tone of the small vessels *Acta Physiol Scand* 17:289-310
- FOLKOW B 1953 A study of the factors influencing the tone of denervated blood vessels perfused at various pressures *Acta Physiol Scand* 27:99-117
- FOLKOW B 1962 Transmural pressure and vascular tone - some aspects of an old controversy *Arch Int Pharmacodyn* 139:455-469
- FOLKOW B 1964 Description of the myogenic hypothesis *Circulat Res* 15 Suppl 1:279-285



- FOLKOW B & ÖBERG B 1961 Autoregulation and basal tone in consecutive vascular sections of the skeletal muscles in reserpine treated cats *Acta Physiol Scand* 53 105-113
- FOLKOW B SONNENSCHEIN R R & WRIGHT D L 1971 Local neurogenic and metabolic effects on precapillary vessels of skeletal muscle *Acta Physiol Scand* 81 459-471
- FRONEK K. & ZWEIFACH B W 1975 Microvascular pressure distribution in skeletal muscle and the effect of vasodilation *Amer J Physiol* 228 791-796
- GASKELL P & BURTON A C 1953 Local postural vasomotor reflexes arising from the limb veins *Circulat Res* 1:27-39
- GRÖNDE P O JÖRHULT J & HELLANDER S 1974 Method for gravimetric registration of changes in tissue volume *Acta Physiol Scand* 91:211-215
- GUYTON A C 1964 In P C Johnson (ed) American Heart Association Monograph No 8 Autoregulation of blood flows *Circulat Res* 15 Suppl 1 278
- GUYTON A C ROSS J M CARRIER O & WALKER J R 1964 Evidence for tissue oxygen demand as the major factor causing autoregulation *Circulat Res* 15 Suppl 1 60-69
- HADDY F J & GILBERT R P 1956 The relation of a venous arteriolar reflex to transmural pressure and resistance in small and large systemic vessels *Circulat Res* 4 25-32
- HADDY F J RICHARDS A G ALDEN J L & VISSCHER H B 1954 Small vein and artery pressures in normal and edematous extremities of dogs under local and general anaesthesia *Amer J Physiol* 176 355-360
- HENRIKSEN O 1976 Local nervous mechanism in regulation of blood flow in human subcutaneous tissue *Acta Physiol Scand* 97 385-391
- JOHANSSON B 1978 Vascular smooth muscle biophysics In: Kaley G and Altura B M (eds) *Microcirculation II* 83-117 University Park Press Baltimore
- JOHANSSON B & BOHR K F 1966 Rhythmic activity in smooth muscle from small subcutaneous arteries *Amer J Physiol* 210 801-806
- JOHANSSON B & HELLANDER S 1975 Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein *Circulat Res* 36 76-83
- JOHNSON P E (ed) 1964 Symposium on autoregulation of blood flow *Circulat Res* 15 Suppl 1 1-291

- JOHNSON P C 1968 Autoregulatory responses of cat mesenteric arterioles measured *in vivo* *Circulat Res* 22:199-212
- JOHNSON M C & HANSON K M 1962 Effect of arterial pressure on arterial and venous resistance of intestine *J Appl Physiol* 17 503-508
- JOHNSON P C & WAYLAND H 1967 Regulation of blood flow in single capillaries *Am J Physiol* 212:1405-1415
- JÖRHULT J & MELLANDER S 1974 Autoregulation of capillary hydrostatic pressure in skeletal muscle during regional arterial hypo- and hypertension *Acta Physiol Scand* 91:32-41
- KJELLMER I 1964 The effects of exercise on the vascular bed of skeletal muscle *Acta Physiol Scand* 62:18-30
- LALONE B, SCHWINGHAMER J & MAJOR T 1975 Autoregulation of skeletal muscle blood flow during pulsatile and non-pulsatile perfusion *Fed Proc* 34 369
- LAWSON H & HOLT J P 1939 Differential manometer method for measurement of blood flow *J Lab Clin Med* 24:639-642
- LUNDVALL J & HILLMAN J 1978 Fluid transfer from skeletal muscle to blood during hemorrhage: importance of beta adrenergic vascular mechanisms *Acta Physiol Scand* 102:450-458
- LUNDVALL J & JÖRHULT J 1976 Beta adrenergic dilator component of the sympathetic vascular response in skeletal muscle: influence on the microcirculation and on transcapillary exchange *Acta Physiol Scand* 96 180-192
- LUNDVALL J & JÖRHULT J 1976 Changes of the pressure drop curve and of resistance along the vascular bed of skeletal muscle evoked by sympathetic stimulation *Microvasc Res* 12:43-54
- LÖFVING B & MELLANDER S 1956 Some aspects of the basal tone of the blood vessels *Acta Physiol Scand* 37 134-141
- MATTHEWS M B C 1964 Muscle spindles and their motor control *Physiol Rev* 44:219-288
- MELLANDER S 1978 On the control of capillary fluid transfer by precapillary and postcapillary vascular adjustments: A brief review with special emphasis on myogenic mechanisms *Microvasc Res* 15:319-330
- MELLANDER S & ARVIDSSON S 1974 Possible 'dynamic' component in the myogenic vascular response related to pulse pressure distension *Acta Physiol Scand* 90:283-285

- MELLANDER S & JOHANSSON B 1968 Control of resistance exchange and capacitance functions in the peripheral circulation *Pharmacol Rev* 20:117-196
- MELLANDER S ÖBERG B & ODELRAM H 1964 Vascular adjustments to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer *Acta Physiol Scand* 61:34-48
- MELSA J L & SCHULTZ D G 1969 *Linear Control Systems* International Student ed McGraw-Hill Book Company
- MYERS H A & HOMIG E R 1969 Influence of initial resistance on magnitude of response to vasomotor stimuli *Amer J Physiol* 216:1429-1436
- ROSS J H FAIRCHILD H M WELDY J & GUYTON A C 1962 Autoregulation of blood flow by oxygen lack *Amer J Physiol* 202:21-24
- SIGURDSSON S JOHANSSON B & MELLANDER S 1977 Rate-dependent myogenic response of vascular smooth muscle during imposed changes in length and force *Acta Physiol Scand* 99:183-189
- SPARKS H V 1964 Effect of quick stretch on isolated vascular smooth muscle *Circulat Res* 15 Suppl 1:254-260
- STAINSBY W M & RENKIN E M 1961 Autoregulation of blood flow in resting skeletal muscle *Amer J Physiol* 201:117-122
- UEHO A & TAKENAKA F 1955 A new measurement of blood flow *Jap J Pharmacol* 4:98-102
- VANHOUTTE P M 1978 Heterogeneity in vascular smooth muscle in Kaley G and Altura B M (eds) *Microcirculation II* 181-309 University Park Press Baltimore





ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 477

**ADAPTIVE CHANGES IN THE SYMPATHETIC  
NERVOUS SYSTEM AND SOME EFFECTOR ORGANS OF  
THE RAT FOLLOWING LONG TERM EXERCISE OR COLD  
ACCLIMATION AND THE ROLE OF CARDIAC SYMPATHETIC  
NERVES IN THE GENESIS OF COMPENSATORY  
CARDIAC HYPERTROPHY**

By

**I ÖSTMAN-SMITH**

OXFORD 1979



**ACTA PHYSIOLOGICA SCANDINAVICA**  
**SUPPLEMENTUM 477**

From the Department of Physiology 1 Karolinska Institutet,  
104 01 Stockholm, Sweden

**ADAPTIVE CHANGES IN THE SYMPATHETIC  
NERVOUS SYSTEM AND SOME EFFECTOR ORGANS OF  
THE RAT FOLLOWING LONG TERM EXERCISE OR COLD  
ACCLIMATION AND THE ROLE OF CARDIAC SYMPATHETIC  
NERVES IN THE GENESIS OF COMPENSATORY  
CARDIAC HYPERTROPHY**

By

**INGEGERD OSTMAN-SMITH\***

\* Present address: Department of Cardiovascular Medicine  
John Radcliffe Hospital Oxford, England



## ACKNOWLEDGEMENTS

I wish to express my deep gratitude to my teacher Dr. Nils O. Sjöstrand, who introduced me to this field for his guidance, encouragement and constructive criticism.

I am further indebted to Professor Ulf S. von Euler and Professor Bengt Andersson for their support and also wish to thank my other co-workers Dr. Göran Svedin and Dr. Henrik Nybläck for their practical help, advice and stimulating discussions. I am very grateful to Professor Lennart Sjöberg for his constructive criticisms and many valuable comments on the manuscript of this thesis. I am also indebted to all members of the staff of the Department of Physiology for their interest in my work and in particular to Dr. Arne Åström for his advice and his generosity in lending me many crucial items of equipment and to Dr. Nils-Åke Persson for technical help with the equipment.

I am most grateful to Dr. M.R. Matthews and Dr. M.E. Florey for carrying out the morphological studies on the sympathetic ganglia.

My warm thanks are also due to Mrs. Kersti Wierling for her skilful and scrupulously reliable technical assistance and to Mrs. Margaret Gurney for typing the manuscript of my thesis.

The work was supported by grants from the Medical Faculty, Karolinska Institutet, Stiftelsen Lars Hiertas Minne and Swedish Medical Research Council (Projects No. B73-04X-3874 and B76-21P-4877-01A) and the generous gifts of Ficoil supplied by AB Pharmacia and of guanethidine sulphate supplied by Ciba are gratefully acknowledged.

Finally, my special thanks to my husband David Smith for his patient support, encouragement and practical assistance.

Apart from previously unpublished results, the present thesis is based on the following papers, which are being referred to by their roman numerals

- I I Östman and N.O. Sjöstrand: Effect of prolonged physical training on the catecholamine levels of the heart and the adrenals of the rat. *Acta physiol. scand.* 1971. 82, 202–208.
- II I Östman, N.O. Sjöstrand and G. Svedin: Cardiac norepinephrine turnover and urinary catecholamine excretion in trained and untrained rats during rest and exercise. *Acta physiol. scand.* 1972. 96, 299–308.
- III I Östman and N.O. Sjöstrand: Reduced urinary norepinephrine excretion during rest, exercise and cold stress in trained rats – comparison between physically trained rats, cold-acclimated rats and warm-acclimated rats. *Acta physiol. scand.* 1975. 98, 209–218.
- IV I Östman: Changed vascular sensitivity to norepinephrine in rats trained by physical exercise. *J. Physiol., Lond.* 1975. 252, 38–39P.
- V I Östman and H. Nybeck: Adaptive changes in central and peripheral noradrenergic neurons in rats following chronic exercise. *Neuroscience* 1975. 1, 41–47.
- VI I Östman-Smith: Prevention of exercise-induced cardiac hypertrophy in rats by chemical sympathectomy (guanethidine treatment). *Neuroscience* 1975. 1, 497–507.

# CONTENTS

CHAPTER 1	HISTORICAL INTRODUCTION	1
CHAPTER 2	GENERAL METHODS	6
CHAPTER 3	EFFECT OF REGULAR PHYSICAL EXERCISE (TRAINING)	12
	Section A. General observations	
	Section B. Effect of chronic exercise on body and organ weight	
	Section C. Catecholamine content in various organs	
	Section D. Urinary excretion of catecholamines and vanillyl mandelic acid	
	Section E. Effect of intermittent exercise	
	Section F. Turnover of catecholamines in various organs	
	Section III. Effect of chronic exercise on effector organ response to catecholamines: study on perfused hindbody and isolated heart	
CHAPTER 4	A COMPARATIVE STUDY OF COLD-ACCLIMATION AND CHRONIC PHYSICAL EXERCISE	53
CHAPTER 5	STUDIES ON THE TURNOVER OF NORADRENALINE IN BRAIN AND HEART BASED ON SYNTHESIS INHIBITION BY $\alpha$ -METHYLTYROSINE	62
CHAPTER 6	SOME OBSERVATIONS ON THE EFFECTS OF CHRONIC SYMPATHETIC DENERVATION BY GUANETHIDINE TREATMENT AND ON THE ROLE OF THE SYMPATHETIC NERVOUS SYSTEM IN CARDIAC HYPERTROPHY	77
CHAPTER 7	SOME WIDER IMPLICATIONS OF THE RESULTS OF THESE STUDIES	92
	SUMMARY OF RESULTS	96
	REFERENCES	102

## Abbreviations used

ACTH	adrenocorticotrophic hormone
BW	body weight
CA	catecholamine
DA	dopamine
ED <sub>50</sub>	the dose that elicits 50% of the maximum response in the effector cell
M <sub>50</sub>	the dose that elicits 50% of the maximal pressure response in the perfused organ
NA	norepinephrine
6-OHDA	6-hydroxydopamine
PNMT	phenylethanolamine N-methyltransferase
RNA	ribonucleic acid
S.D.	standard deviation
S.E.	standard error of the mean
SIF cell	Small Intensely Fluorescent cell
VMA	vanillyl mandelic acid
f	degrees of freedom
k	rate constant
	number of observations
n.s.	not significant
t <sub>1/2</sub>	half-life
	variance



## HISTORICAL INTRODUCTION

## EXERCISE AND ORGAN SIZE

In 1748 Robinson in his dissertation on the subject of comparative anatomy pointed out that species with a normally high degree of physical activity had larger heart weight relative to their body weight than was found in those species whose natural habits involved lower degrees of physical activity. His observations were confirmed and extended by Bergmann, Bollinger and Becher and Perrot in the late 19th century (quoted by Kulbs, 1906). Furthermore, it was observed that within a species individual animals that were subjected to heavy physical exertion, for example race horses and draught dogs (Bollinger, 1896) and draught oxen and hounds (Friedberger and Frohner, 1896 quoted by Kulbs, 1906) showed an increased heart size. In man Herachien (1898) commented on cardiac hypertrophy occurring in cross-country skiers and Schaeffer (1907) showed that regular bicycle riding for three years leads to an increase in heart size.

The first controlled experimental study of the effect of regular physical exercise on the heart seems to have been performed by Kulbs (1906) who exercised two young dogs on a kind of a treadmill for 3-6 months with a final daily exercise load of 3 hours per day. When he compared the hearts of these young dogs with matched control animals whose physical activity had been restricted, the trained dogs were found to have a 52-83% increase in heart weight. Kulbs also later observed that the older the animal the smaller is the degree of cardiac hypertrophy obtained (Kulbs, 1912) an observation that has recently been confirmed in the rat by Bloor and Leon (1970). Based on studies on the heart size of wild rabbits, trapped and subsequently kept under conditions that restricted physical activity, Kulbs also stated that exercise induced cardiac hypertrophy can regress (Kulbs, 1912) and this has subsequently been confirmed in rats by Hort (1951) and Leon and Bloor (1968). Likewise Jundell (1912 quoted by Becher, 1923) suggested that the cardiac hypertrophy seen in athletes normally regresses when regular training is abandoned and this was documented in a longitudinal study on a cross-country skier by Dedichen (1920).

Experimental studies have established that regular physical exercise causes cardiac hypertrophy in numerous species such as the dog (see Kulbs, 1912; Steinhaus *et al.*, 1932), the pig (Kulbs and Barbinch, 1906 quoted by Kulbs, 1912), the rat (Hazel, 1915; Becher, 1921 and number of later authors) and the rabbit (Hiratsuki, 1919).

Already in 1912 Kulbs found that there was no great change in the proportion of the total heart weight contributed by each individual heart chamber in dog hearts that had become hypertrophied following regular physical exercise. This was confirmed for the exercise-induced cardiac hypertrophy of the rat heart by Hort (1951) and Van Lere *et al.* (1966).

However, the heart is not the only organ that shows adaptive changes following regular exercise. skeletal muscle mass is increased (Morpurgo, 1897; Kulbs, 1912; Petow and Seibert, 1925; Hort, 1951) and the amount of subcutaneous fat is decreased (Kulbs, 1906, 1912; Stevenson *et al.*, 1965; Ocan and Holloszy, 1969). With moderately heavy physical exercise body weight may remain unchanged (Kulbs, 1906; Hata, 1915; Petow and Seibert, 1925) but with fairly intensive exercise the body weight of the trained animal becomes lower than that of control animals (Bazník and Sertády, 1924; Meyer *et al.*, 1954; Whitworth and Grummege, 1956; Van Lere and Northup, 1957; Grimm *et al.*, 1963; Leon and Bloor, 1968; Ocan and Holloszy, 1969).

Hatal (1915) noted that there was an increase in the weight of the adrenal glands in his spontaneously running female rats whereas the increase in the male rats was not significant. Subsequently the weight of the adrenal glands of rats has been found to be increased by exercise in rats of both sexes (Baznak and Serkady 1934; Hort, 1951; Frenki and Casley 1962; Buuck and Tharp, 1971; and later authors).

The weight increase of the adrenals can be accounted for by the increase in size of the adrenal cortex (Baznak and Serkady 1934; Buuck and Tharp 1971) and this in turn is largely due to increase in width of the zona fasciculata (Hort, 1951; Buuck and Tharp 1971).

The occurrence of adrenal cortical hypertrophy is not surprising as it has been shown that physical exertion leads to secretion of adrenocortical hormones. This was first suggested by the decrease in cholesterol content of the adrenal cortex following exercise described by de Jongh and Rosenthal (1933); subsequently physical exertion was found to lead to the excretion in the urine of increased amounts of adrenocortical hormones (Venning and Kazmin 1948) and finally it was shown that blood levels of corticosteroids in the rat particularly corticosterone were increased by exercise in man (Froesch *et al.*, 1964 and later authors) in dogs (Nezar 1971) as well as in rats (Frenki *et al.* 1968; 1969; Buuck and Tharp, 1971; for extensive references see Nezar 1971).

The increase in size of the adrenal glands occurs within two weeks of the start of regular exercise (Baznak and Perjés, 1935; Hort, 1951; Frenki and Casley 1962) and the size of these glands begins to regress upon cessation of exercise (Baznak and Perjés, 1935; Hort, 1951).

The influence of regular exercise on the size of the spleen has been the subject of conflicting reports. Thus Kribe (1906-1912) found no consistent change in the dog as did Hort (1951) in the rat, whereas Hatal (1915) found a smaller spleen in his exercised rats. Petow and Siebert (1926), also using rats, found a lower splenic weight only in the group that ran slowly to 320 m/day, not in the group that ran at a higher speed for 20 min/day although the latter group had the more pronounced degree of cardiac hypertrophy.

## THE INFLUENCE OF ACUTE EXERTION ON THE SYMPATHO-ADRENAL SYSTEM

The first observations on the role of the sympatho-adrenal system in exercise appears to have been the demonstration by bio-assay on the denervated iris by Hartmann *et al.* (1922) of the release of adrenaline into the blood during muscular work and this observation was confirmed on the denervated heart by Cannon *et al.* (1924). Wade *et al.* (1935) studied the secretion of adrenomedullary hormones in the venous effluent from an extirpated adrenal gland in conscious dogs subjected to exercise and found that only strenuous work, but not running of moderate intensity led to significant increase in secretion of medullary hormone. That muscular work leads to an increased excretion of noradrenaline (NA) in the urine was first shown by bio-assay by Holts *et al.* (1947).

It has been concluded that adrenaline present in the urine mainly originates from the adrenal medulla whereas the NA excreted in the urine largely is derived from sympathetic nerve-endings (Euler *et al.* 1954). The first study that related the urinary excretion of both NA and adrenaline to the intensity of exercise was performed by Eder and Haltnier (1952). They found in men that exercise of moderate intensity with an oxygen consumption not exceeding 2 l/min caused only a small increase in the amount of adrenaline excreted in the urine whereas at high work loads the excretion of adrenaline in the urine was increased 5-20 fold. Likewise excretion of NA in the urine was moderately increased at moderate work rates whereas at high work loads the urinary excretion of NA was increased up to 10 fold.

Vandeklu (1960) found that in venous plasma the increases in absolute concentrations of catecholamine (CA) with increasing work-load were smaller for adrenaline than for NA, particularly at high work-loads. However if the increases are expressed in relation to the resting concentrations then his data actually show that in males at work-load of 800 kpm, the plasma concentration of adrenaline is 3-fold greater than at rest whereas that of NA is only 2-fold greater. At a higher work-load of 1200 kpm the concentrations of both adrenaline and NA were 5.6-fold higher than at rest. A similar trend was seen in the females studied by Vandeklu. Thus it appears that, relative to rest, there is greater change in the adrenaline concentration than in the NA concentration at low work-loads. It is striking that at high work-loads there was no change in the relative proportion of adrenaline to NA in plasma compared with the proportion in plasma taken at rest. Furthermore data has been presented by Higgendel *et al* (1970) suggesting that NA levels in arterial plasma increase exponentially with increasing work load and are similar in different individuals if the relative work load is the same.

The difference in the degree of activation of the sympathetic nervous system and the adrenal medulla has been attributed to their different actions, the sympathetic nervous activity being involved in the cardiovascular readjustments to exercise and the prevention of hypotension resulting from regional vasodilatation whereas the secretion of adrenaline from the adrenal medulla is thought to be mainly triggered by the accompanying elements of emotional stress and by exercise-induced hypoglycaemia (see reviews by Euler 1974 and Frankenhaeuser 1971).

During exercise the increase in cardiac output, necessary to supply the increased amounts of oxygen required by the working muscles, is achieved mainly by increasing the heart rate whereas the stroke volume during supine exercise only increases by about 10–20% (see review by Bevegard and Shepherd, 1967). Although the Frank-Starling mechanism contributes to the beat to beat regulation of cardiac output the large alterations in cardiac output induced by exercise are therefore achieved by the autonomic nervous system.

The increase in heart rate occurring during exercise can, in a normal heart, be assumed to be due largely to increased activity in cardiac sympathetic nerves, rather than to increased concentrations of circulating catecholamines or to decreased vagal tone for the following reasons. (i) There is normally a simultaneous increase in myocardial contractility which would not be expected to occur after simple vagal inhibition. (ii) Vagal blockade alone does not achieve maximal heart rate and does not increase cardiac output, furthermore the mechanical systole is longer and stroke volume is smaller than when the same heart rate is achieved by exercise. Also the maximal heart rate achieved through exercise is not influenced by atropine (see review by Bevegard & Shepherd, 1967). (iii) Although both circulating NA and adrenaline have positive inotropic and chronotropic actions, the experiments of Donald and Shepherd (1964) on greyhounds with surgically denervated hearts demonstrated that the increase in cardiac output achieved in the denervated heart by circulating catecholamines was of slower onset than that seen in intact dogs and was qualitatively different, with smaller increase in heart rate and a larger increase in stroke volume from the changes found in unoperated dogs at the same cardiac output (Donald and Shepherd 1964).

Evidence consistent with increased sympathetic nervous activity in the heart of rats during exercise was produced by Gordon *et al* (1966<sup>b</sup>) who found that after inhibition of NA synthesis with  $\alpha$ -methyl para-tyrosine the NA levels of the heart fell significantly more rapidly in rats subjected to exercise than in sedentary rats, implying greater rate of turnover of NA in the exercised animals.



## MODIFICATIONS IN THE SYMPATHO-ADRENAL SYSTEM INDUCED BY PHYSICAL TRAINING

Euler and Helmer (1962) in their study on urinary excretion of catecholamines in men following exercise observed that one of their subjects an athlete considerably better trained than the other subjects showed in contrast to the other individuals no increase in the amount of NA excreted in the urine at work load intensities up to an oxygen uptake of 2.1 l/min. The role of physical fitness in the activation of the sympatho-adrenal system during muscle work remained controversial however since Vandal (1960) did not find any significant differences in the plasma levels of NA in physically fit group compared with less fit subjects working at the same work load while on the other hand Carlsson *et al* (1968) in a study on mentally disordered patients, found that the plasma NA levels at a given work load were lower in patients who had been subjected to physical training.

It has long been established that athletes display a phenomenon called relative bradycardia with a slower heart rate and larger stroke volume both at rest and during any given work load and as the maximum heart rate is the same they also have a higher maximum cardiac output (Dedichen 1920 Herschler 1921 Kaul 1926 Bock *et al* 1928 Hoogwerf 1929 Schneider and Crumpton, 1940 and later authors see review by Sevegard and Shepherd 1967). The relative bradycardia can develop after a fairly limited period of vigorous training both in humans (Kneib *et al* 1942) and in man such as the rat (Holt, 1951 Tipton 1965 Buuck and Tharp 1971). The bradycardia develops roughly in parallel with the development of cardiac hypertrophy (Tipton 1965 Buuck and Tharp 1971). Since stimulation of the vagus nerve causes cardiac slowing, an obvious hypothetical explanation of the relative bradycardia of the athletic heart was that athletes developed an increased vagal tone at least in the efferent fibers to the heart. This hypothesis has become very popular (Kaul 1926 Steinhaus, 1933 Melnikowicz 1956 Hennrich *et al* 1960). However the experimental evidence is not at all convincing. Herschler (1921) quotes several studies showing that 1 mg of atropine sulphate causes an increase in heart rate of between 20-80 beats/min in the general population and then reports his study on athletes where this dose in spite of causing dryness of the mouth lead only to a minimal acceleration of heart rate about 5 beats/min and in some individuals had no effect on heart rate at all. He concluded that since the bradycardia of the athletic heart could not be abolished by atropine it was not due to increased vagal tone. Likewise Kaul (1926) found no cardiac acceleration in athletes after administration of 1 mg of atropine sulphate but he interpreted the lack of effect of atropine to be due to massive vagal tone that could not be overcome by usual doses, and he was able to show that after 2 mg of atropine there was some cardiac acceleration in athletes although only about 70% of the heart rate increase seen in his control group after half the dose.

Since atropine sulphate crosses the blood-brain barrier, it is possible that the higher dose of atropine used by Kaul entered its effect through actions on the central nervous system. The latter possibility is supported by the finding of Robinson *et al* (1953) that 2 mg of atropine sulphate caused restlessness in the experimental subjects and lead to an increase in the  $O_2$ -consumption at rest. Furthermore there are several observations suggesting that increased vagal tone alone cannot explain the bradycardia in athletes. Firstly as discussed by Reindets *et al* in his review (1954) the athletic heart classically displays both prolonged isometric period of the systole and prolonged total systole and vagal stimulation or acetylcholine administration cannot therefore alone simulate the cardiovascular characteristics of athletes. Secondly Raab *et al* (1960) were not able to abolish the difference in mean heart rate between a physically active group of men and relatively inactive control group by fairly large doses of atropine sulphate (0.025 mg/kg) around 1.75 mg/person. Thirdly Tipton (1965) showed that

relative bradycardia develops in response to training in rats that have had their right or left vagus cut, and that atropine causes less cardiac acceleration in trained than in untrained rats (Tipton and Taylor 1966).

Herrlich *et al.* (1960) base their proposal of excessive vagal tone on findings of increased acetylcholine and/or acetyl cholinesterase levels in the heart, but, as will also be illustrated in Sections C and F (Chapter 3) on the NA stores of the heart, organ transmitter concentration does not always parallel transmitter turnover and thus these findings do not in themselves indicate an increased parasympathetic tone.

An alternative explanation for the bradycardias of the athlete's heart would be that rather than an increase in vagal tone there was instead a decrease in nervous activity in the sympathetic nerves innervating the heart. In a study specifically designed to look at the role of cardiac sympathetic nerves in the bradycardias of the athletic heart, De Schryver *et al.* (1967) claimed that the reduction of heart rate was due to depletion of NA, however the training intensity used in this study was too low (see Section B, Chapter 3 for further discussion) to produce the usual criteria for successful training effect such as cardiac hypertrophy and reduction in body weight. In summary, therefore, as concluded also by Åstrand and Rodahl (1970) the origin of the relative bradycardias in the athlete remains obscure.

## AIMS OF THE PRESENT STUDY

In 1932 Cannon wrote: "The man who daily takes a cold bath and works until he sweats may be keeping fit because he is not permitting a very valuable part of his bodily organization (the sympatho-adrenal system) to become weakened and inefficient by disuse."

However, although there has been a lot of experimental research studying the changes in activity of the sympatho-adrenal system during various forms of physical and emotional stress, in 1969 comparatively little was known about the effects of chronic alterations in the activity of the sympatho-adrenal system. Chronic exposure to cold had been shown to lead to acclimation involving among other things increased sensitivity to calorogenic effects of NA, lower excretion of NA in the urine during cold exposure and a larger capacity to survive cold exposure (note see Introduction to Chapter 4). Chronic exposure to "emotional" stress had been studied by Welch and Welch in spontaneously fighting mice and found to lead to increases in NA content in brain and heart and increases in adrenal adrenaline content (Welch, 1967; Welch and Welch, 1969). However, there did not appear to have been any studies on the effect of chronic physical stress on the activity of sympathetic nerves in various organs, and of the secretory activity of the adrenal medulla when the animal was at rest and undisturbed.

The main aims of the present thesis were therefore to study the following questions:

1. Does the sympatho-adrenal system adapt to a chronic increase in its activity?
2. If so — how is this adaptation expressed?
3. How does adaptation to chronic stress compare with acclimation to cold?
4. What is the role of cardiac sympathetic nerves in the relative bradycardias of the athletic heart?
5. What is the role of the cardiac sympathetic nerves in the development of exercise-induced cardiac hypertrophy?

## GENERAL METHODS

## EXPERIMENTAL ANIMALS

The animals used in most of the experiments were male albino rats of the Sprague-Dawley strain, obtained from Anticimex AB, Stockholm. In one experiment, male Wistar rats from Charles River Ltd, United Kingdom, were used. The rats were kept at room temperature unless otherwise stated, and had free access to water and food (standard chow for rats). They were kept 4 to 6 rats (depending on age and size) to a cage of 55 by 37 cm.

## TRAINING METHODS

*Running*

The experiments where the animals were trained by running were performed using a motor-driven treadmill. As the rats would not run spontaneously throughout whole training session and some rats developed very expert technique in effortlessly sliding along comfortably resting against the back wall of the treadmill. It was necessary to discourage the rat from touching the back wall. This was achieved with the help of an electric fence on the back wall of the treadmill which intermittently delivered weak electric shocks. Following such negative inducement the rats quickly learned to keep running at a steady pace to avoid contact with the back fence. After about 4 weeks adaptation to the treadmill actual training was started at a speed of 22 m/min for 1 h/day and the duration and intensity of the exercise session were gradually increased, as described in detail in paper I, so that during the last 5 weeks the rats ran at 37 m/min for two 40 min sessions with a 10 min period of rest in between covering a total distance of 2,840 m/day.

*General comments on treadmill training.* The inbred albino rat is not a very good runner for its size. Furthermore, the occasional weak electric shock received by the poor runner adds an undesirable element of "emotional" stress to a study on exercise. Towards the end of the study when the exercise load was high it was occasionally noted that shock made the recovering rat so aggressive as to start fight with another rat in the treadmill. This was however by no means a regular feature.

*Swimming*

Since treadmill running did not seem to be a very satisfactory method for the regular long term exercise of rats it was decided instead to try regular swimming as an alternative exercise method.

The water temperature was kept at  $35 \pm 1^\circ\text{C}$ . Most authors using swimming as a means of exercise use water temperatures between  $33^\circ\text{C}$ – $35^\circ\text{C}$  (Crews and Aldinger, 1967; Lin and Horvath, 1972; Leon *et al.* 1975) and Lin and Horvath (1972) state that rats maintain a normal body temperature during swimming at  $35^\circ\text{C}$ ; furthermore Tan *et al.* (1954) showed that water temperature of about  $36^\circ\text{C}$  is optimal for an untrained rat (the minimal swimming time getting shorter at temperatures both above and below). The fact that a water temperature slightly below body temperature is optimal is probably because it allows the animal to get rid of the excess heat produced by muscular work without actually becoming hyperthermic.

When a rat is put into water to swim for the very first time it appears at first surprised and usually just floats on the surface trapped in the fur for a moment, following which it rapidly discovers how to swim about by propelling itself with its feet. Subsequently it swims rapidly around the edges of the bath for a couple of minutes apparently searching for a way to climb out. When this search has proven futile an untrained rat then settles for the minimum level of exertion necessary "treading water" to keep afloat.

Before training was started with long daily sessions, the rats in the training group were accustomed to water over a period of about five days: the first day each rat was left only 5 min in the water; the second day 15 min and thereafter increasing by 15 min/day until a daily session of 1 h/day was reached. After 4 weeks of training at this load the daily swimming periods were stepwise increased by 30 min at a time until a final daily exercise period of 2 h or, in papers IV and V, 2.5 h was reached. In the swimming baths measuring (length  $\times$  width  $\times$  height) 70  $\times$  44  $\times$  53 cm, 8–10 rats swam together in each bath (paper II); in the baths measuring 100  $\times$  70  $\times$  60 cm (papers III–VI) 12–15 rats swam simultaneously.

Following each swimming period the rats were transferred to "drying cages" containing several layers of soft blotting paper where they immediately proceeded to shake off the water and started drying their fur without showing any signs of excessive fatigue. After about 20 min, their fur was virtually dry and they were transferred back to their ordinary cages where they immediately started eating. In contrast to our rats trained by treadmill running which never eat shortly after the exercise presumably due to running-induced hyperthermia, in the studies on NA turnover and urinary excretion of catecholamines during and after exercise in papers II, III and VI, the controls and trained rats swam together in the same bath during the acute exercise session. In this way the swimming activity becomes subjectively uniform in the two groups.

*General comments on swim training:* Having experience of both methods of training rats described above it is my firm opinion that, perhaps surprisingly, swim training is the method which involves the least "emotional" stress. The initial alarm reaction in the rat when left in the water, as described above, soon disappears as the rat gets accustomed to the water and the fact that it will inevitably be picked up again. Subsequently the rats merely start swimming about at a fairly leisurely pace and sometimes spontaneously dive and swim about under water to explore the bottom of the tank.

Rats practically never aspirate water during training sessions as also indicated by the fact that there was no morbidity whatsoever in chest infections (which might have been expected to occur from aspiration of the bath water that was always somewhat contaminated by fecal material). Furthermore, among the total of 170 rats trained by daily swimming in the various experiments there was not a single fatality due to drowning.

#### *Cold-exposure experiments*

*Cold acclimation* was achieved by keeping rats for 14 weeks at a constant temperature of  $4 \pm 1^\circ\text{C}$ , in plastic cages furnished by shavings with 4–5 rats per cage and with food and water *ad libitum*. Illumination essentially followed normal office hours. During acute cold exposure rats were kept, for 22 h at  $4^\circ\text{C}$ , singly without any bedding, in metabolic cages for urine collection and with free access to food and water.

### Activity studies

Spontaneous physical activity was measured by a photoelectric (Motron) activity meter with a photo cell floor under fairly intensive illumination during daytime.

### Preparations of organs and tissue extracts

Rats were killed by exsanguination under light ether anaesthesia. Organs were quickly dissected out and carefully trimmed and the heart cut open to expose both ventricles and carefully blotted before weighing. Brains, hearts, spleens and submandibular glands were homogenized with an Ultra-Turrax apparatus in 15 ml ice-cold 0.4 M perchloric acid containing 0.1 mM ascorbic acid. The homogenates were centrifuged to sediment the insoluble matter and in most studies the supernatant was titrated to pH 3.8 with KOH and recentrifuged for the elimination of potassium perchlorate and the supernatant was kept frozen at  $-20^{\circ}\text{C}$  until the assay was performed. Catecholamines were adsorbed on alumina, eluted and oxidized according to Cheng (1964) or according to Euler and Lishajko (1961) as described in detail by Swedin (1971). In the turnover study using  $\beta$ -methyldopamine (Chapter III) it was necessary to use the Cheng (1964) method, since the Euler-Lishajko (1961) method gave falsely high results.

The recovery figures for NA added to random samples and carried through the extraction and oxidation procedures were  $84.4 \pm 3.8\%$  in heart homogenates,  $89.3 \pm 2.3\%$  in spleen homogenates and  $77.1 \pm 5.1\%$  in submandibular gland homogenates. Data given in the papers are uncorrected for recovery and expressed as  $\mu\text{g}$  free base. Extracts from adrenal glands were oxidized according to Euler and Lishajko (1961) or Cheng (1964) directly without prior adsorption to alumina. In the study published in paper V the supernatant of perchloric acid organ homogenates was adsorbed on alumina columns, from the eluate tyrosine was isolated on Amberlite CG 120 columns and in the eluate from the alumina column NA and dopamine (DA) were separated using Dowex 50W-X4 columns. All procedures were as described in detail by Nybeck (1971). DA was assayed according to the method described by Carlsson and Waldeck (1958) and Carlsson and Lindqvist (1962) and NA according to Cheng (1964). The tyrosine content in the Amberlite eluate was determined according to Wong *et al.* (1964). In paper V the recovery of exogenous radioactive NA and DA added to brain extracts before the passage over the various columns as described was 50% for NA and 42% for DA.

*Urine collection and assay of urinary catecholamines and VMA.* Urine was collected in metabolic cages where the animals were kept individually over a period of 22 h. Contamination of collected urine by food and fecal particles were minimized in paper II by the use of glass drop-bed below the funnel and in paper III and VI by using glass fibre wool as a filter in the funnel. When urine collection was performed after swimming session the rat was lifted out of the bath manually blotted with blotting paper and an attempt was made to initiate reflex emptying of the urine bladder by suprapubic pressure. The latter procedure was never successful possibly because the bladder was already empty. Rats do defecate in the water while swimming and may void urine then too. The collection bottles contained, at the start, enough 1M HCl (4–6 ml) so keep the final pH to 4 or below. After removal of the rats, the funnels of the metabolism cages were rinsed with 10 ml of 0.1M HCl and the rinsing fluid that filtered through was included in the sample. The pH values of all urine samples were checked and virtually all samples had pH considerably below 4.0; the results of those samples where the pH exceeded 4 (usually due to heavy leakage of the water bottle in the cage) were discarded. Urine was centrifuged

for 15 min at 9000 r.p.m., the supernatant was titrated to pH 3.8 with 2M KOH and recentrifuged and the supernatant kept frozen at  $-20^{\circ}\text{C}$  until the assay was performed.

For the assay of catecholeamines one tenth volume of 0.27 M disodium EDTA was added to the urine sample which then passed through an alumina column that was eluted as described by Svedén (1971). The NA and adrenaline content was assayed according to Euler and Lishajko (1961) or Cheng (1964) and is expressed as  $\mu\text{g}$  free base.

Vanillylmandelic acid (VMA) was estimated by the method of Plesno *et al.* (1962) which involved oxidation with sodium periodate. 7 ml of urine was shaken with ca. 0.5 g of Florisil and the VMA in 0.5 ml of the supernatant was extracted and oxidized as described by Plesno *et al.* (1962). Excess periodate was removed by adding glycerol. The reaction product was extracted into toluene and the optical density at 360 m $\mu$  was measured. Because of variation between the amount of contaminating material present, blank tube for each sample of urine was prepared in which an excess of glycerol was added before the periodate. Internal standards, containing 10  $\mu\text{g}$  of VMA, were done for each set of oxidations.

Sympathetic ganglia were homogenized in 0.3 M sucrose and tyrosine hydroxylase activity was assayed according to Nagatsu *et al.* (1964) using 3,5-[ $^3\text{H}$ ]-L-tyrosine.

#### Isolated organs

**Isolated heart.** Half an hour before the experiment the rat was pretreated with 800 I.U. heparin intraperitoneally. The animal was anesthetized with an intraperitoneal injection of sodium pentobarbitone (Mebumal sodium) 8 mg/100 g B.W. The beating heart was excised with one cut and immediately transferred to ice-cold 0.9% (w/v) NaCl which stopped the heart beating within a few seconds. After a quick rinse in saline the cut end of the aorta was identified and any large bulk of extraneous tissue excised. The aorta was cannulized and retrograde perfusion of the heart by the Langendorff procedure was commenced following which the heart resumed beating almost immediately. At the same time 5 ml of heparin solution (10 mg/ml dissolved in the perfusion medium) was slowly injected in the perfusion cannula. Any remaining extraneous tissue was trimmed away and free outflow from pulmonary artery was secured.

The perfusion medium was Krebs-Henseleit bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ ) with the addition of 14 mM glucose, 0.5 mM of disodium ethylenediamine tetra-acetate (EDTA) titrated to pH 7.40 and 0.11 mM ascorbic acid. The perfusion medium was oxygenated by gas mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  and had been equilibrated with the  $\text{O}_2$ - $\text{CO}_2$  mixture at room temperature for at least 20 min before the perfusion was commenced. The pH of the equilibrated medium with additions was 7.40. The heart was perfused by the perfusion fluid at  $38^{\circ}\text{C}$  (higher temperatures increased the liability to arrhythmias) and was surrounded by a water jacket maintained at  $38^{\circ}\text{C}$ , with the top sealed by Perspex. The perfusion fluid was delivered to the heart at a constant rate via peristaltic pump and there was no recirculation of used Krebs solution. As the hearts of trained rats on the average are about 7% heavier than those of control rats it was necessary to have a correspondingly higher perfusion rate for the hearts of trained rats, in order to achieve the same average perfusion rate in flow per g heart. The ideal flow rate was considered to be that which gave an average initial perfusion pressure of about 60 mm Hg in both experimental groups, as 60 mm Hg was considered by Neely *et al.* (1967) to be the optimal perfusion pressure for isolated hearts. In pilot experiments a flow rate of 17.2 ml/min was found to be

suitable for control hearts and a flow rate 18.5 ml/min for hearts of trained rats, which resulted in an average flow of 13.4 ml/min/100 g heart in both experimental groups. The perfused hearts were weighed after perfusion and it was found that the average heart weight of the two groups did not differ significantly from the expected ratio as the mean heart weight in the trained group was 8% higher than that in the control group. The perfusion pressure at the start of the determination of dose response curves averaged  $66 \pm 7$  mm Hg in the control group and  $70 \pm 8$  mm Hg in the hearts from trained rats. The perfusion pressure usually rose somewhat during the experiment averaging 85 mm Hg in both experimental groups at the end of the experiment.

Left ventricular contractions were recorded with a rubber balloon inserted via the mitral valve and inflated *in situ* with a pressure of 5 cm H<sub>2</sub>O. The intra balloon pressure was recorded via Statham pressure transducer.

Initially I attempted to obtain a dose-response curve via infusion of NA at a constant rate by an automatic syringe maintaining each infusion rate until a steady state heart rate was obtained. This however proved to be impossible in practice since it induced a very high rate of arrhythmias. It was therefore necessary to give each dose as a 10 second period of infusion of NA at constant concentration and to allow the heart to recover between each dose. The dose given in the dose response curve is that infused during the 10 seconds.

During each experiment the temperature of the perfusion fluid, the perfusion pressure, the left ventricular balloon pressure and the heart rate was continuously recorded on Grass Polygraph Recorder and the rate of perfusion was monitored by measuring the effluent from the heart.

**Perfused hindbody** The perfused hindbody of the rat was prepared as described by Folkow *et al* (1970) with only minor modifications. Rats were treated with 500 I.U. heparin i.p. 30 min before the experiment and anaesthetized with 8 mg/100 g BW of sodium pentobarbitone (Mebromel). The rat was exsanguinated, with care being taken to tie off all severed vessels, and the aorta was cannulated above the iliac anastomosis as described by Folkow *et al* (1970). The vena cava was opened wide to provide free outflow. After the hindbody had been isolated from the upper part with standardized mass ligatures it was cut away from the upper half and the hindfeet and tail were tied off to minimize cutaneous circulation. The isolated hindbody was placed in a plastic chamber with a drain for the outflow heated by a 38°C water jacket and covered by plastic film to keep temperature and humidity constant. The hindbody was perfused by Krebs-Henseleit's buffer (the same as used for the isolated heart) with the addition of 5.5 mM of glucose, 0.11 mM ascorbic acid and 4% (w/v) of Ficoll (a synthetic colloid, a polymer of sucrose and epichlorohydrin) with average molecular weight of 77 000 kindly supplied by Pharmacia Fine Chemicals. In a second series 2  $\mu$ M disodium EDTA was added as well. The perfusion medium was oxygenated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> gas mixture and when equilibrated the pH of the medium including additives was 7.40. The hindbodies of both groups were perfused at the same rate averaging between 10–11 ml of perfusate/100 g tissue each minute and the perfusion fluid was kept at 38°C. However unlike Folkow *et al* (1970) only one hindbody was perfused at a time. As described by Folkow *et al* (1970) slug injections of 1–3 mg papaverine was given initially to ensure maximal vasodilatation; however there was never any further drop in peripheral resistance following these injections. The papaverine was allowed a 20 min wash out period before the NA infusion was commenced. The NA was infused using motor driven syringe at constant rate that was increased in a stepwise fashion and the perfusion pressure was continuously recorded via Statham pressure transducer – at the aortic level. At high concentrations of NA there was not infrequently a small initial overshoot in perfusion pressure before stabilizing at a slightly lower value. In all such instances the steady-state

pressure was used in the calculations of the dose-response curve. Towards the end of the experiment, there was as described by Folkow *et al* (1970) some tissue oedema despite of the use of colloids, but there was no difference between the two groups in this respect.

## DRUGS

L-noradrenaline bitartrate (Sigma chemicals) and *dl*-isoprenaline sulphate (Abbot Laboratories) were used and concentrations calculated as  $\mu\text{M}$  base. DL-4-hydroxy-3-methoxy-mandelic acid (Sigma Chemicals) was used as standard in the VMA assay. For the infusion solutions, high concentrations of noradrenaline was dissolved in 0.9% NaCl with 0.11 mM ascorbic acid and 1.0 mM EDTA in order to obtain maximal stability of the catecholamine. Isoprenaline was dissolved in 0.9% NaCl with 0.11 mM ascorbic acid.

## STATISTICS

Significance of difference between the means of various groups were calculated with the help of Student *t*-test. Hal-Hives and significances of differences between rate constants were calculated using conventional statistical methods (Edwards, 1954; Goldstein *et al* 1962).



# EFFECT OF REGULAR PHYSICAL EXERCISE (TRAINING)

## SECTION A GENERAL OBSERVATIONS

### EXERCISE TOLERANCE AND SWIMMING BEHAVIOUR

As judged by their behaviour untrained rats when swimming 8 rats per bath are moderately fatigued after 2 h of swimming, fairly exhausted after 4 h of swimming and very exhausted after 8 h of continuous swimming. The trained rats on the other hand looked very little affected even after 8 h of swimming (paper I). It is clear from simple observation that the greater the number of rats per unit surface area in the bath the greater the swimming activity and therefore it is important for such data to be included in publications describing studies on swim training.

When trained and untrained rats are exercised in equal numbers in separate swimming tanks, it is noticeable that the swimming activity of the trained rats is higher than that of untrained rats as the trained rats spontaneously swim about whereas the untrained rats tend to "breed water" a lot of the time. This difference in activity appears to be eliminated when trained and untrained rats are put in the same tank in reasonable numbers and such procedure is therefore an essential precaution in any study directly comparing trained and untrained rats during swimming.

### *Aggressive behaviour during exercise*

When under stress male rats frequently start to fight each other. This was not infrequently observed in the rats during forced running in the treadmill toward the end of the longer exercise sessions.

The incidence of stress-induced aggressive behaviour was much lower during swim training than during treadmill running: it only occurred in one out of four studies: the one described in paper IV where the density of rats in the bath was somewhat higher than the others. Even in this study only 3 out of 72 rats displayed an aggressive behaviour and when these rats were isolated in a separate bath this behaviour subsided and there were no further problems. These 3 rats were all included in the group studied 6 months after cessation of exercise.

### *Behaviour after exercise*

After a long session of forced running rats salivated profusely drank a lot but exhibited decreased spontaneous motor activity and did not eat for at least half an hour. In contrast after swim exercise the trained rats immediately proceeded to groom themselves and subsequently started to eat. This differential behaviour is probably largely due to the hyperthermia induced by running, self-ventilation being an important part of thermoregulation in the rat (Hansmworth, 1967; Ekstrom, 1974).

There was a subjective impression that the spontaneous motor activity of resting chronically exercised rats was slightly higher than that of control. This impression was however not verified in studies in illuminated photocell-type activity cages where the spontaneous activity of both groups of rats was found to be the same.

## SECTION B EFFECT OF CHRONIC EXERCISE ON BODY AND ORGAN WEIGHT

(Papers I, II, III, V, VI)

## RESULTS

*Body weight*

In all studies using albino rats, regardless of the type of exercise used, chronic physical exercise was found to decrease the rate of weight gain during growth without influencing body length, so that at the end of the training period the body weight was from 5 to 8% lower than in controls (papers I, II, III, V).

*Heart*

In all the studies on chronically exercised animals the hearts were found to have hypertrophied. The degree of cardiac hypertrophy was remarkably constant in all studies irrespective of the type of training and variations in duration of training, being 7%, measured as absolute heart weight, in the studies described in papers I, II, III and V.

*Adrenal glands*

Adrenal hypertrophy was most pronounced in rats trained by forced running, with weight increases of 31% (paper I) but was significant also in swim-trained rats ranging from +8 to +10% (papers II and III).

*Spleen*

In paper II the spleen weights of chronically exercised rats were found to be on average 2% lower than those of controls but this difference was not significant; in paper V however the 9% decrease in splenic weight of the trained group was statistically significant ( $p < 0.01$ ).

The weights of *brain* and *submandibular glands* remain unaltered in rats after chronic physical exercise by swimming (paper V, III).

6 months after cessation of chronic exercise there is no significant difference between body weight, adrenal weight and splenic weight between swim-trained rats and controls (paper III and unpublished observations). Although the absolute heart weight was 5% higher in trained rats this is no longer significantly different from controls, but the heart ratio in trained rats remains very significantly higher than the control value (10%,  $p < 0.001$ ). In this case the fact that the difference in absolute heart weight is no longer significant is due to the increasing individual variation in size with increasing age. This is so because even if the contribution of the non-significant -3% difference in mean body weight between the two groups is allowed for, the difference in heart ratio still remains statistically significant.

## DISCUSSION

*Body weight*

That rats subjected to heavy chronic physical exercise gain less weight than control rats was pointed out already by Baznek and Berkady (1934) and subsequent authors (see Chapter 1). This effect is not always seen with low-intensity exercise; however, prolonged (Pitow and Seibert, 1925) and at least in adult rats, is often only seen after daily and not after intermittent exercise (see Section E). The decreased weight gain seen in rats subjected to daily exercise is due to a relative hypophagia on the day of exercise. Meyer *et al.* (1954) using treadmill running found that light exercise did not increase food intake but actually decreased it below control values, whereas with increasingly long daily exercise sessions the rats would increase their food intake but never enough to compensate fully for the increased energy need and consequently the body weights of the trained rats always remained below control values. The depression of food intake is not solely due to hyperthermia induced by muscular exertion since it is also present in rats which are made hypothermic by swimming in water at 30 °C (Stevenson *et al.* 1966) although to a lesser degree than in the rats exercised by treadmill running. The resulting decrease in body weight is due to a reduction in white fat tissue which is not fully compensated for by the increase in weight of skeletal muscle occurring in trained rats (references see Chapter 1) and thus the proportion of the body weight constituted by fat decreases in parallel with increasing exercise load (Stevenson *et al.* 1966; Hanson *et al.* 1967). After single bouts of exercise however, there is a compensatory increase in food intake during the following two days (Edholm *et al.* 1955; Thomas and Miller 1958; Stevenson *et al.*, 1966); this explains the lack of effect on body weight after intermittent training.

The effect of exercise on body weight appears to be less marked in female than in male rats (Van Liere and Northrup 1957). Richter (1968) found that untrained female rats subjected to extremely long periods of swimming (40–80 h) subsequently developed a syndrome characterized by hyperphagia and obesity as well as pseudopregnancy. These observations could explain why Crews and Aldinger (1967) who subjected female rats to stressful daily swimming for 6 h/day found no effect on their body weight.

There is no evidence that the effect of exercise on body weight is due to increased thyroid activity as mentioned above trained rats display relative bradycardia and as shown in Section G of this chapter the intrinsic heart rate of isolated hearts is not altered, whereas it is well known that hyperthyroidism causes an increase in the intrinsic heart rate of the isolated heart (see review by Fredberg and Hamolsky 1974).

*Heart*

That daily physical exertion leads to adaptive growth of the heart has been shown for many species and many different forms of exercise (see Chapter 1 for references). This adaptive hypertrophy does not require the presence of either growth hormone or thyroxine or any other pituitary factor as demonstrated by Tipton and Tchong (1971), who found that forced running still produced cardiac hypertrophy in hypophysectomized rats. Development of adaptive growth of the heart in response to chronic exercise does however seem to require the presence of cardiac sympathetic nerves since it is abolished after chemical sympathectomy (paper VI; see Chapters 6 and 7 for further discussion).

Exercise-induced cardiac hypertrophy involves equally both the right and left ventricles (references

see Chapter 1) and leads to an increase in both the longitudinal and transverse diameters of the heart (Hort 1951) and thus also in total volume (Crews and Aldinger 1967). Malik *et al.* (1974) who studied the compensatory hypertrophy in dogs after chronic pressure over-load (experimental aortic stenosis) and after chronic volume over-load (chronic arterio-venous fistula) found that whereas the left ventricular wall thickness was increased in both types of hypertrophy only chronic volume over-load produced an increase in the internal diameter of the left ventricle. Since the increase in cardiac work during exercise is mainly due to increased cardiac output, the findings by Malik *et al.* (1974) in the high output state are probably relevant to discussion of exercise-induced changes. The finding that athletes have larger stroke volume than untrained subjects not only during rest but throughout exercise and up to the maximal heart rate (Bock *et al.* 1928 T. Spstrand 1955 Wang *et al.* 1961 Bevegard 1962 Bevegard *et al.* 1963 Astrand *et al.* 1964) could be explained either by increased sympathetic nervous activity (causing a shift in the Frank-Starling relationship) or by an increase in internal ventricular dimensions as a result of exercise-induced cardiac hypertrophy. Our findings of a lower level of sympatho-adrenal activity in trained rats, compared with controls during exercise as well as rest (see Sections D & F) would, if it is also true in man, mean that the greater stroke volume of athletes is due to change in the dimensions of the ventricle.

The increase in heart weight seen following chronic physical exercise is not due to an increase in cardiac muscle mass alone as there is an increase of collagen content parallel to that in heart weight (Bartolomeo *et al.* 1969). Furthermore exercise leads to the formation of new capillaries (Mandache *et al.* 1972 Ljungqvist and Unger 1972 Mandache *et al.* 1973) with resultant increase in capillary/fibre ratio (Thorner 1935 Petrin *et al.* 1936 Petren and Syken, 1937 Leon and Bloor 1968 Bloor and Leon, 1970 Ljungqvist and Unger 1972) and the dimensions of coronary and extra-coronary anastomosing arteries also increases (Leon and Bloor 1968 Bloor and Leon, 1970). In the rat some experimental conditions like altitude (Suzmore *et al.* 1973) carbon monoxide exposure (Pannay *et al.* 1974) chronic isoprenaline administration (Stanton *et al.* 1969 Le Blanc *et al.* 1972) and experimental hyperthyroidism (see review by Korecky and Beznak 1971) can lead to massive hypertrophy with increases of 50–70% in the weight of one or both ventricles of the heart in a relatively short space of time sometimes only 10 days. In man prolonged exposure to pathological conditions can lead to increases of 100% and more in the weight of the heart (Brown 1971). In comparison with these pathological changes, the degree of exercise-induced cardiac hypertrophy in these studies (7 to +6%) (papers I–VI and Section E) looks quite modest but it is of the same magnitude as that seen in another physiological cause of cardiac hypertrophy cold-accclimation (see Chapter 4). It is also of the same magnitude as the exercise-induced cardiac hypertrophy reported by those authors who specify that the hearts are trimmed and blotted before weighing (Tipton and Tchang, 1971 +6% Leon *et al.* 1975: +6%) the importance of these procedures are obvious in view of the increased vascularity and thereby blood content, seen in exercise-induced cardiac hypertrophy (see above).

There appears to be universal agreement that in the rat the cardiac hypertrophy seen after relatively short fasting exposure to various pathological conditions is reversible. Thus cardiac hypertrophy due to renal hypertension (Hall *et al.* 1963) hyperthyroidism aortic coarctation and nutritional anaemia (Beznak *et al.* 1969) isoprenaline treatment (Stanton *et al.* 1969), or simulated altitude (Suzmore *et al.* 1973), regresses on removal of the provoking stimulus. This regression is a rapid process with half life of approximately 7 days (Stanton *et al.* 1969 Suzmore *et al.* 1973). In view of this it is somewhat surprising that cardiac hypertrophy +6% in terms of absolute heart weight, still persists as long as 6 months after cessation of training (paper III). Other investigators (Scherer 1923 Hort 1951 Leon and Bloor 1968 Ljungqvist and Unger 1972 and Unger *et al.* 1973) have reported a rapid return of heart

weight to control values when chronic exercise is discontinued. In paper III young male rats were exercised, while Hort (1951) used old male rats and Ljungqvist and Unger (1972) and Unger *et al.* (1973) used relatively adult female rats. Male rats continue to grow throughout their life-span (in contrast to female rats) and in my rats the training period coincides with puberty and the phase of rapid growth of the male rat. The importance of exercise during adolescence and puberty for the development of cardiovascular dimensions in man is well known (see Astrand and Rodahl 1970) and it has been shown that young male rats need less exercise to develop cardiac hypertrophy than adult males, and that they develop a greater degree of cardiac hypertrophy with about the same training intensity (Bloor and Leon 1970). Comparing paper III with the study of Leon and Bloor (1968) who also used young male rats, the training program in paper III is found to be more severe with longer period of training and with a final daily swimming time twice as long; perhaps this could account for the difference between my results and those of Leon and Bloor. The study of Secher quoted above suffers from lack of age-matched controls which makes his conclusions, based on heart ratio, unreliable since the heart ratio in the young rapidly growing male rat decreases with increasing age (see control values in Grimm *et al.* 1963; Buuck and Therp 1971; Therp and Buuck, 1974).

Why then should cardiac hypertrophy induced by exercise in young, still growing, animals persist whereas that induced by the various pathological conditions mentioned above regresses? In adult animals cardiac muscle cell division does not normally occur (Erikhine 1968; Saeki *et al.* 1968; Shafiq *et al.* 1968) and the number of cardiac muscle cell nuclei in the adult rat is not altered by chronic exercise (Hort, 1951). Thus in the adult animal hypertrophy of cardiac muscle is produced by increased fibre diameter and, when the internal dimensions of the heart are increased, presumably also by increase in length of the individual muscle cell just as normally occurs during postnatal growth; this is accomplished without an increase in length of the individual sarcomeres (Li *et al.* 1963). Mitoses of cardiac muscle cells do however occur in young rats, the rate of mitosis being quite high at birth and subsequently decreasing with increased age. In Sprague-Dawley rats the rate of cardiac muscle cell mitosis is still significantly elevated at the age of 2 months and reaches adult "values" sometime before the age of 4 months (Klinge and Stocker 1968). Furthermore Rumyantsev and Ilinskij (1968) found that in young male albino rats experimental infarction of the left ventricle, leading to mitral regurgitation, could lead to 100-fold increase in mitotic index in cardiac muscle cells in the left auricle. Such post-natal mitosis does not, however, usually lead to cytoplasmic division (Klinge and Stocker 1968). The possible occurrence of postnatal mitosis in the young rat has to be considered as one explanation for the discrepancy between the workers that claim that exercise-induced cardiac hypertrophy regresses completely (refs. see above) and the finding of persistent cardiac hypertrophy as long as 6 months after cessation of training reported in paper III. If a detailed comparison is made with the studies of Ljungqvist and Unger (1972) and Unger *et al.* (1973) who used the same strain of rats (Sprague-Dawley) as was used in paper III it is found that whereas the rats in paper III were about 6-7 weeks of age at the start of the experiment, and thus young enough to still have significant occurrence of mitosis in cardiac muscle cells (Klinge and Stocker 1968) the female rats used by Ljungqvist and Unger (1972) and Unger *et al.* (1973) were about 10-11 weeks old at the onset of training, and seem to have been past the age where mitosis in cardiac muscle cells can occur, since no mitoses were observed in their rats, neither in control rats, nor in groups with cardiac hypertrophy of various origins (Mendelsohn *et al.* 1973). It is tempting, therefore, to speculate that the failure of the exercise-induced cardiac hypertrophy to regress after cessation of training in paper III is due to the presence of an increased number of cardiac muscle cell nuclei and thus the connection it is noteworthy that the absolute increase in heart weight over the 6 months following cessation of training is virtually

the same in both groups, +0.33 g in control rats and +0.32 g in co-trained rats. In view of these considerations and in view of the findings of Bloor and Leon (1970) already mentioned above, that very young male rats more readily develop cardiac hypertrophy than adult rats, it would seem possible that in the very young animal hyperplasia of cardiac muscle fibres or possibly occurrence of binucleate cells, as well as hypertrophy may contribute to the adaptive growth of the heart.

Finally I would like to make some comments about the mode of expression of results when studying cardiac hypertrophy. It has been claimed that when animals lose weight rapidly following starvation or undernutrition the heart weight decreases roughly in proportion to the decrease in body weight (Jackson 1925; Van Lere and Sleeth 1936; Bazmak, 1964) but this has been shown not always to be the case (Odeh and Holloszy 1970). Since the expression of heart weight as fraction of body weight, for example g heart weight/100 g BW makes it easier to obtain statistical significance as the inter-individual variation is considerably decreased the use of heart ratio has become popular in the study of cardiac hypertrophy of various origins. This procedure is however only justified when the age and the mean body weight of the groups compared are the same. This is so for the following reasons. Firstly the heart ratio is not constant from birth but rather starts at a high value and then gradually decreases (see control values Grimm *et al.* 1963; Buuck and Therp 1971; Therp and Buuck, 1973) in at least some rat strains. A plot of ventricular weight versus body weight will fall on a straight line from the age when the male rat has reached a body weight of about 200 g or more but, due to the slope of the line the heart ratio still decreases with increasing age (see Grimm *et al.* 1963). Thus it is not justified to draw conclusions about, for example regression of cardiac hypertrophy on the basis of heart ratio values alone as done by Secher (1923) and Ljungqvist and Unge (1972) in the absence of age-matched controls for comparison. Secondly treatments leading to changes in body weight without actually changing body length do not always lead to parallel changes in heart weight as shown by Odeh and Holloszy (1970) who by inducing weight loss caused marked increases in heart ratio in animals where the absolute heart weight either was unchanged or actually decreased. This point is also illustrated by the increased heart ratio but unaltered heart weight and body length found in rats chemically sympathectomized by guanethidine treatment (see paper VI). Clearly claims of cardiac hypertrophy should only be based on increased size of the heart itself and not on results on heart ratio inflated by loss of body fat!

#### *Adrenal glands*

As reviewed in Chapter 1 the increase in weight of the adrenal glands in chronically exercised animals can be accounted for by the increased secretion of ACTH during exercise sessions which induces corticosteroid secretion and leads to an increase in thickness of the fasciculate zone of the adrenal cortex. In view of the increases in the adrenal content of both adrenaline and NA seen in chronically exercised rats (see Section C) the possibility that there is an increase in number and/or size of individual chromaffin cells in the adrenal medulla must also be considered since mitoses of chromaffin cells have been demonstrated in the adrenal glands of adult rats (Malvalde *et al.* 1966) although they are normally very infrequent.

The finding that guinea pigs exercised by forced running for five months did not have hypertrophied adrenal glands (Oscman and Sjostrand, 1971) is somewhat surprising in view of the evidence of increases in corticosteroid secretion during exercise in various species (see Chapter 1). It is still conceivable that the lack of adrenal hypertrophy could be due to species difference since the guinea pigs are better adapted to running and will keep running at considerably higher speeds than rats do

(unpublished observation). It is perhaps more likely that they have adapted so fully to the exercise situation that the "stress" of one *semo* per day does not produce a sufficient increase in ACTH secretion to give rise to an increase in the weight of the adrenal cortex. It is noteworthy that these guinea pigs had been subjected to regular exercise for a period of five months much longer than the period of training in most studies. In this context some longitudinal studies are of interest and give some support to the above hypothesis. The difference in plasma corticosterone levels between exercised rats and controls reaches a maximum some time between 2 and 4 weeks after initiation of training and subsequently decreases again so that resting plasma levels of corticosterone are normal 6 to 8 weeks after the onset of regular exercise although at that stage adrenal cortical hypertrophy persists (Frankl and Carley 1962; Sharp and Buuck 1974). When trained and untrained animals are submitted to the same work-load the blood level of steroids increases more in the untrained than in the trained rat and the same is true for humans (Frankl *et al.* 1968, 1969). Furthermore plasma levels of corticosterone stay elevated after exercise for much longer in untrained (more than 3 h) than in trained (45 min) rats (Frankl *et al.* 1968). All these studies suggest that the exercise-induced secretion of ACTH, and thereby the stimulus for adaptive growth of the adrenal cortex, is highest early on during the training period, and subsequently decreases considerably and perhaps in the "ideally trained" state disappears completely. A further observation that suggests that the guinea pigs were not subject to much "stress" is the fact that they did not have significantly increased catecholamine content in the adrenal gland (Jørgensen and Sjostrand, 1971).

As regards the argument of which type of exercise treadmill running or swim training is the less stressful form of exercise the finding that the adrenal hypertrophy is so much greater in the rats exercised on the treadmill than in the swim-trained rats gives further support to the suggestion (see Chapter 2) that in rats swim training is the form of chronic exercise that involves least stress.

#### Spleen (papers III and V)

Alterations in the weight of the spleen following chronic physical exercise appear to have been previously reported only by Hatae (1918) and Petow and Seibert (1928). The former found a decrease in size of the spleen in exercised rats and the latter authors reported 10% lower spleen weight in rats that ran for 320 min/day at a speed of 6.8 m/min, whereas there was no change in splenic size in rats running 20 min/day at 36 m/min. Petow and Seibert (1928) did not comment on this finding however. There appear to be two possible explanations for a change in weight of the spleen. Firstly since the spleen is a blood-rich organ it is possible that unequal degree of vasoconstriction in the different groups of rats could lead to an apparent change in organ size. Secondly the amount of actual tissue stroma, most notably lymphoid tissue, can be decreased. Considering the first alternative it is theoretically possible that this could account for the difference between controls and trained rats since vascular smooth muscle in trained rats exhibits an increased sensitivity to the vasoconstrictor actions of NA (paper III, Section G). On the other hand the impulse activity of sympathetic nerves in the spleen as estimated by the turnover of NA, is significantly lower in trained rats than in the controls. There appears to be no reason to expect that the method of killing used in paper V (decapitation) should cause more marked increases in nervous activity of sympathetic nerves to the spleen in trained rats than in controls and thus the first explanation of the decrease in spleen weight is unlikely to be correct. Furthermore the exercise-induced decrease in spleen weight is seen also in chemically sympathectomized rats (paper VI). The second alternative, decrease in lymphoid tissue, seems to have more arguments to support it.

This regular administration of steroids has been found to lead to a decrease in splenic weight (Mokumut *et al.* 1990 Whurman 1986) due to a decrease in lymphoid tissue. This mechanism could then explain the decrease in weight of the spleen found in chronically exercised rats since there is much evidence that physical exertion leads to increased secretion of corticosterone in rats (see above). Such a mechanism could also account for the otherwise puzzling results of Petow and Siebert (1925) since the 20 min period exercise might have been too short to produce high levels of corticosterone in the plasma.

Salivary glands will be discussed in Chapter 4

## SECTION C: CATECHOLAMINE CONTENT IN VARIOUS ORGANS

(Papers I, II, III, V unpublished observations)

### RESULTS

#### Brain

In the brains of chronically exercised rats, killed 48 h after the last period of exercise both the NA content and the NA concentration were consistently and significantly increased. In one group, killed after 17 weeks of daily swim-training the NA content of the whole brain was 25% higher than that of sedentary controls ( $p < 0.001$  paper VI). In another group which was studied after 14 weeks of daily swimming, the NA content in the brains of trained rats was increased by 14% ( $0.73 \pm 0.02 \mu\text{g NA/g brain}$ ,  $n = 12$ , as compared to  $0.64 \pm 0.02$  in controls,  $n = 11$ ,  $p < 0.01$ ). The DA content of the brain, on the other hand, was not significantly altered (paper VI).

#### Heart

Total cardiac NA content has been found to be increased after chronic exercise (papers I: 14%, n.s., II: 25%,  $p < 0.001$  III: +26%,  $p < 0.01$  V: +22%, n.s.) but the increase was only significant in swim-trained rats and not in the study using treadmill running (paper I). The concentration of NA in the heart was likewise increased, although to a somewhat smaller extent due to the concomitant cardiac hypertrophy (papers I: +6%, n.s., II: 16%,  $p < 0.01$  III: 18%,  $p < 0.05$  V: +7%, n.s.).

#### Salivary glands

NA content and concentration in submandibular glands were not significantly altered (paper III).

#### Spleen

The total NA content of the spleen was increased by 26% after 14 weeks of swim-training (paper III,  $p < 0.01$ ) no significant increase was however found in the study after 17 weeks of swim-training (paper VI).



### Adrenal glands

The adrenal NA content was increased in the trained animal in all the studies, but the increase was more pronounced in the studies utilizing swim-training (papers I: +94%,  $p < 0.001$  III: +32%,  $p < 0.01$ ) than in the study using treadmill running (paper II: +24%, *n.s.*). The adrenal adrenaline content was increased by a similar degree in all the studies on trained rats, whether trained by running or by swimming (papers I: +21%,  $p < 0.01$  II: +20%,  $p < 0.05$  III: +13%,  $p < 0.01$ ). In swim-trained rats, but not in rats trained by running on a treadmill the increase in NA content was relatively greater than the increase in adrenal ne content, so that there was an increase in the proportion of catecholamines comprised by NA (paper I:  $22 \pm 1\%$  compared to  $15 \pm 1\%$  in the controls  $p < 0.001$  paper II:  $16.1 \pm 0.7\%$  as compared to  $13.9 \pm 0.7\%$ ,  $p < 0.05$ ).

Thus, in chronically exercised rats the stores of transmitter are increased in noradrenergic neurons in brain, heart and possibly spleen, and in the chromaffin cells in the adrenal medulla.

### DISCUSSION

The effects of chronic exercise on the amounts of CA in various tissues can best be interpreted in terms of the general hypothesis that chronic increase in the activity of a neuron leads to changes in its biochemical composition some of which may be truly adaptive in the sense that they enable the neuron better to fulfill its physiological function. One of the earliest demonstrations of this principle were the morphological studies of motor and sensory neurons subjected to a sustained increase in impulse flow which showed that there was an initial depletion of Nuel substance (RNA + protein) followed by morphological and biochemical evidence of increased nuclear and nucleolar activity (Hydén 1943). After prolonged increase in impulse flow in chronically stimulated vestibular neurons, Hydén and co-workers demonstrated an increase in RNA and protein content and in cytochrome-oxidase and succinoxidase activity (see review by Hydén 1967). Maeno and Krotchina (1966) found that neurons of the intramural ganglia in the heart undergoing hypertrophy following experimental aortic stenosis as reflected by their morphological appearance appeared to undergo a similar sequence of events terminating in a stage of "stable hyperfunction" with an hypertrophied neuron with increased total content of Nuel substance. In cholinergic neurons it has been suggested that an increase in the level of choline acetyltransferase activity reflects increased neuronal activity (Oesch, 1974 see also Ekström 1975). However, the biochemical events that follow increase in afferent nerve impulse flow have been studied in greater detail in sympathetic neurons and in particular in the adrenal gland.

### Adrenal gland

**Enzymes of catecholamine biosynthesis.** In the adrenal gland the activities of three of the enzymes involved in CA biosynthesis have been shown to be increased after various stressful stimuli. The enzymes are tyrosine hydroxylase the presumed rate-limiting step (Bartley *et al.* 1960; Lavitt *et al.* 1965), dopamine  $\beta$ -hydroxylase characteristic component of adrenergic storage vesicles (Smith, 1972), and phenylethanolamine N-methyltransferase (PNMT) the enzyme converting NA to adrenaline. Rows the activities in all three enzymes occur in cold stress (Thoenen, 1970; Kvetnansky *et al.* 1971<sup>a</sup>), immobilization stress (Kvetnansky *et al.* 1970-1971<sup>b</sup>) as well as in "psychosocial stimulation" (Henry *et al.* 1971). Similarly increases in tyrosine hydroxylase and dopamine  $\beta$ -hydroxylase activity in the adrenal gland have been shown 24-96 h after insulin-induced hypoglycaemia (Viveros *et al.* 1969).

Patrick and Kirschner 1971) PNMT was not studied in these experiments. All these are conditions that increase splanchnic nerve impulse flow (see below). The increase in tyrosine hydroxylase activity can be prevented by denervation of the adrenal gland, but can be induced in a denervated adrenal by means of repeated acetylcholine administration (Patrick and Kirschner 1971). Furthermore the long-term rise in tyrosine hydroxylase activity occurring after prolonged (at least 4 h) increase in splanchnic nervous activity is evident after 24 h but reaches maximum after 48 h and is inhibited by blocking transcription and/or protein synthesis (see reviews by Thoenen *et al* 1973 and Kirschner 1975). Consequently this increase in tyrosine hydroxylase activity is presumed to be induced by increased pre-synaptic release of acetylcholine and be due to *de novo* synthesis of enzyme. Increase in dopamine  $\beta$ -hydroxylase activity in adrenal glands also appears to be predominantly regulated by increase in splanchnic nervous activity and be mediated through *de novo* protein synthesis, although ACTH may serve permissive role (see review by Axelrod, 1972). PNMT however seems to be controlled by steroids from the adrenal cortex (Wurtman and Axelrod, 1965; Wurtman 1968) as well as by neural factors (Ra *et al* 1971; Kvetnansky 1973).

*Conditions leading to increases in adrenal catecholamine content.* Increased adrenal adrenaline content has been observed in various chronic physiological and emotional stress situations such as cold acclimation (Refs. see Chapter 4) grouping (Welch and Welch, 1968; Welch and Welch 1969), daily fighting (Welch and Welch 1968) and "psychosocial stimulation" (Henry *et al* 1971) all these are conditions which on acute exposure are known to increase urinary adrenaline excretion and/or cause acute decrease in adrenal adrenaline content (cold see Chapter 4; grouping: Welch and Welch, 1968; fighting: Welch and Welch 1969).

Similarly increased adrenal NA content has been reported in some but not all studies on cold acclimation (for references see Chapter 4) and after "psychosocial stimulation" (Henry *et al* 1971).

Thus there is abundant evidence that increased afferent nervous activity in the adrenal medulla causes adaptive responses in the chromaffin cell leading to increased synthesis capacity (as indicated by increased activity of tyrosine hydroxylase and PNMT) as well as possibly an increased storage capacity (as indicated by increased dopamine  $\beta$ -hydroxylase activity) for the catecholamines.

It can be concluded that the observed increases in adrenal CA content in chronically stressed animals is an adaptation to the increased secretory activity of the adrenal medulla. That trained rats have an increased storage capacity for CA in their adrenal glands is also corroborated by Roffman *et al* (1973) who found that repeated swimming sessions lead to an increase in adrenal dopamine  $\beta$ -hydroxylase activity.

*Exercise and adrenal catecholamine turnover.* That adrenal adrenaline synthesis and turnover are increased during prolonged exercise in rotating drum has been shown previously (Gordon *et al* 1966<sup>b</sup>) but there seems to have been no studies on adrenal NA turnover during exercise. It is well known that urinary NA, as well as adrenaline excretion (Refs. see Section D) and plasma NA levels (see review by von Euler 1974) are increased during exercise but in view of the concomitant increase in sympathetic nervous activity the adrenal contribution can only be assessed by direct turnover studies. However, the increase in adrenal NA content found in the swim-trained animals (papers I, II, III) suggest that adrenal NA turnover is also significantly increased at least during strenuous physical exercise perhaps as secondary defence when the transmitter stores in peripheral sympathetic nerves threaten to get exhausted as occurs in the heart in untrained rats after prolonged swimming (paper II).

*Treadmill running compared with swimming.* There is a somewhat different pattern of increase in adrenal CA content in the rats trained on a treadmill (paper I) compared to rats trained by swimming (papers II-III) since in the rats trained by swimming there was a significant increase in the proportion of the catecholamines comprised by NA. This difference in the response of the adrenal medulla to the different types of training could be due to the fact that running on a treadmill with an electrical fence certainly would appear to constitute much more of an emotional stress than swimming does while the swim-training used on the other hand probably constitutes a greater working load. It is well known that "emotional" stress is the more potent stimulus for adrenal adrenaline secretion (Kvetnansky and Mukulaj 1970 see review by Frankenhaeuser 1971). The absence of significant increase in adrenal adrenaline content seen in guinea pigs trained by treadmill running (Ostman and Sjöstrand, 1971) could be due to species differences or to the fact that they were less emotionally stressed by the running than the rats were since an electrical fence was not used and since they are physically much better adapted to running than rats are. A third possibility since the guinea pigs were trained for a longer period of time than any rat group (22 weeks as compared to 14 to 16 weeks) and attained higher increases in cardiac NA concentration (21% as compared to 6%, 16% and 16% in rats) is that as the peripheral sympathetic nervous system gets progressively more adapted to the demands of exercise there is progressively less need for supplementary adrenal secretory activity. It is noteworthy that in the guinea pigs there was also an absence of adrenal hypertrophy (Ostman and Sjöstrand, 1971).

## Brain

Many experimental stimuli, that during acute exposure increase the turnover of NA in whole brain or part of the brain (for references see section F and paper VI), when intense cause an acute decrease in the NA content of the brain. This has been shown with severe cold stress (Linn and Maynard, 1962; Maynard and Linn 1964; Stone 1970) with forced running (Gordon *et al.* 1966<sup>b</sup>; Stone 1971) grouping of previously isolated mice (Welch and Welch 1966; Welch 1967<sup>a</sup>) aggregation of "strangers" (Bliss and Ailion 1966) and in fighting mice (Bliss and Zwenzger 1966; Welch and Welch 1969). However, when the stimulus is chronic, or intermittent but repeated regularly over a prolonged period of time, an elevation of NA content in the brain occurs, as seen in prolonged cold exposure (Ingenito and Bonnycastle 1967) after two weeks exposure to daily fighting (Welch and Welch, 1969) after prolonged "grouping" (Welch 1967<sup>b</sup>) and after repeated electroconvulsive shocks (Kury *et al.* 1967). Thus the finding of an elevated NA content and concentration in the brain of chronically exercised rats fits well with the changes reported in both physiological and "unphysiological" types of stress. It should be emphasized that whole brain NA content is a very crude measure and that the elevation of NA content in the brains of chronically exercised rats may be limited to one or a few areas of the brain, perhaps the hypothalamus and/or the brain stem, the areas that show increased NA turnover during exercise (Stone 1971; Gordon *et al.* 1966<sup>b</sup>).

The underlying mechanism of this increase is likely to be the induction, presumably secondary to increased impulse flow in the neuron, of the enzymes of the biosynthetic pathway for the transmitter as also occurs in peripheral noradrenergic neurons and the adrenal medulla (see above for references). Thus prolonged cold exposure leads to an increase in tyrosine hydroxylase activity which initially is seen only in the medulla oblongata (Thoenen 1970) where the cell bodies occur of many of the noradrenergic neurons that send terminals to the hypothalamus (Dahlstrom and Fuxe 1966 see review by Bolme *et al.* 1972) which is one area where there is a pronounced increase in NA turnover during cold stress (Simmonds 1969). After a time lag, presumed to be due to axonal transport of the enzyme

there is an increase in tyrosine hydroxylase activity in other brain areas as well (see Costa and Meek, 1974). Tyrosine hydroxylase activity in the brain has also been shown to be increased by other conditions leading to increased NA content in the brain such as electroconvulsive shock (Muscicchio *et al* 1969) and immobilization stress (Lamprecht *et al* 1972). However the best indication of increased storage capacity is an increase of dopamine  $\beta$ -hydroxylase activity. It would be of interest to study dopamine  $\beta$ -hydroxylase activity in the brain in physiological conditions which elevate brain NA content, but this seems not to have been done as yet. However chlorpromazine treatment which is known to lead to increased NA turnover in the brain (Corrodi *et al* 1967; Nyback and Bedvall 1970) has been shown to increase dopamine  $\beta$ -hydroxylase activity in the rat brain upon chronic administration (Wee *et al* 1974).

The finding that the DA content in whole brain is not increased in chronically stressed rats is not surprising since the synthesis (Gordon *et al* 1966<sup>b</sup>) and turnover (Ostman and Nyback, previously unpublished, see Section F) of DA in whole brain has not been found to be increased by physical exercise.

## Heart

*Induction of enzymes and increased storage capacity* The increases found in both the content and concentration of NA in the heart are likely to be the result of an adaptive response to daily periods of markedly increased nervous activity just like the adaptive responses of the brain and in the adrenal medulla. Marked increase in cardiac sympathetic activity as estimated by NA turnover studies, has been shown to occur both during running (Gordon *et al* 1966<sup>b</sup>; Sheldon *et al* 1976) and during swimming (paper II) and nerve impulse-induced increases in tyrosine hydroxylase levels after combined cold and swimming stress takes place in rat stellate ganglia and heart just as in the adrenal medulla (Thoenes *et al* 1972). Furthermore Roffman *et al* (1973) have shown that chronic, but not acute swimming stress leads to increased dopamine  $\beta$ -hydroxylase activity in cervical sympathetic ganglia. Increased tyrosine hydroxylase activity in the heart would suggest that there is increased maximal synthesis capacity in the "athletic heart". Paper II provides some support for this assumption as the trained rats were able to maintain their (higher) stores of NA throughout 8 h of continuous swimming, with an absolute turnover of NA  $1.0164 \mu\text{g NA/heart/h}$  during the 4–8 h period whereas the untrained rats were unable to maintain their cardiac stores of NA during the same interval. As the turnover was higher in the untrained rats they would have needed  $0.236 \mu\text{g NA/heart/h}$  to maintain their stores but after 4 h the maximal synthesis rate evidently was only  $0.146 \mu\text{g NA/heart/h}$  and consequently there was drop in cardiac NA content. It can be seen therefore that  $0.164 \mu\text{g NA/heart/h}$  was submaximal synthesis rate for trained rats, whereas in untrained rats synthesis rate of  $0.146 \mu\text{g NA/heart/h}$  was an absolute maximum.

As mentioned above dopamine  $\beta$ -hydroxylase is almost exclusively confined to noradrenergic storage vesicles, and rise in the level of dopamine  $\beta$ -hydroxylase would thus seem to indicate the presence of an increased number of NA storage vesicles or perhaps less likely an increase in dopamine  $\beta$ -hydroxylase content per storage vesicle.

*Does reformation of nerve terminals occur?* Whether the storage vesicles are contained within the same number of terminals or whether the number of noradrenergic terminals is increased as well is not yet absolutely clear. Unger *et al* (1973) reported a normal number of noradrenergic axons but an increased number of noradrenergic varicose fibers in the hearts of chronically stressed rats. They

suggested that this increase was related to the well-known increase in capillary density in the heart of chronically exercised animals (see Section B for references) because each cardiac capillary is normally accompanied by at least one noradrenergic nerve fibre (Unge *et al.* 1973). This proposal fits with what is known about the capacity of the noradrenergic neuron to extend its field of innervation (see Burnstock and Costa 1975) and with the finding that, at the site of capillary neoformation in the hearts of exercised rats there are varicose nerve fibres in close proximity to the dividing pericyte (Unge *et al.*, 1973). (In this connection it is of interest that Sm th and Wolpert, 1975, have suggested that the presence of nerves may actually be necessary for the formation of new blood vessels.)

Since especially in the atrium, the number of noradrenergic varicose nerve fibres considerably exceeds the number of capillaries (Dehlstrom *et al.* 1966) one would not expect the same proportional increase in NA content as in capillary density. Thus, with swimming schedule less severe than ours Leon and Bloor (1968) and Bloor and Leon (1970) found increases in capillary/fibre ratio of 40–44% in swim-trained rats.

Further support for the concept of neoformation of adrenergic nerve terminals adaptive cardiac hypertrophy is the report by Borchard (1978) that in hypertrophied human myocardium the density of nerve terminals is higher than would be expected if unaltered number of terminals was simply diluted by hypertrophied muscle cells.

It can be concluded that the rise in the content and concentration of NA in the hearts of chronically exercised rats may well be accounted for by an increase in the number of noradrenergic nerve terminals each with perhaps relatively normal content of storage vesicles.

*Factors determining the degree of increase in cardiac noradrenaline content.* It is noteworthy that there does not seem to be a very close correlation between the degree of cardiac hypertrophy and the degree of increased cardiac NA content in all our training experiments where rats were studied (papers I, II III V Section E) the cardiac hypertrophy obtained was constantly from 7 to +8%, whereas the increase in total NA content in the heart varied twofold between 14% and 26%. It seems that the increase in cardiac NA levels obtained with regular physical exercise is related to several factors described below.

First, the intensity of the precipitating stimulus. Thus, the cardiac hypertrophy obtained in the cold-acclimated rats was the same 7%, as in the exercised group but the increase in total NA content in the heart was only +16% compared with +26% in the exercised group (paper III see Chapter 4).

Second, the daily work load. Thus Leon *et al.* (1975) who like us used young male rats but swam them for 1 h/day only 5 days/week for 12 weeks found no significant increase in total cardiac NA content (+5%) but a virtually identical degree of cardiac hypertrophy (+8%).

Third, the frequency of the stimulus. Thus, intermittent swim-training, 3 times/week but with the same daily swimming time leads to smaller increase in total cardiac NA content in spite of the same degree of cardiac hypertrophy (+8.5%) (see Section E in this chapter).

Fourth, possibly also the age of the animals. The failure to obtain a significant increase in cardiac NA content in the run-trained group could be related to the fact that not only is the exercise probably less intense than the swim-training but also to the fact that these rats were somewhat (3 weeks) older as well in this respect it is of relevance that Bloor and Leon (1970) found that the same type of swim-training caused a comparatively larger increase in ventricular dry weight and in capillary/fibre ratio in one-month old than in three-month old rats (see also Section B).

*Results of other workers.* Our findings on the cardiac NA content and concentration in chronically exercised rats would seem to be at variance with the work of De Schryver and co-workers (De Schryver *et al* 1967 1969 De Schryver and Mertens-Smythagen 1972) who claim that the bradycardia of the athletic heart might be related to their finding of decreased NA concentration in the hearts of "exercised" rats. Their exercise program is however of very low intensity (running speed of 8.3–12.8 m/min (about one third to one fourth of the running speed used by other workers studying the athletic heart (Becher 1921 1923 Hort 1961 Van Lier and Northup 1957 paper I) and they exercise their rats only three times per week. Such training parameters are not sufficient to induce cardiac enlargement (Petow and Siebert, 1926 Leon and Bloor 1968 Bloor and Leon 1970 Hecht *et al* 1973) and thus it is hardly surprising that De Schryver and his associates never find any cardiac hypertrophy in their "trained" rats. Since the athletic heart by definition is an enlarged heart, De Schryver and co-workers have obviously not been studying this condition but a different experimental situation. Furthermore since the occurrence of significant bradycardia in trained rats seems to be associated with the occurrence of cardiac hypertrophy (see Chapter 1) and since De Schryver *et al* never actually measured the heart rate in their rats their conclusion — "this decrease of sympathetic transmitter substances might explain in part the bradycardia found in the athletic heart" (De Schryver *et al* 1967 1972) must be rejected.

However their report itself that during their experimental conditions with such mild and only intermittent exercise program they see decrease both in total content and in concentration of NA, lasting up to 6 days after cessation of exercise seems very puzzling, particularly since they report that, in contrast three days after single bout of exercise in untrained rats their cardiac NA concentration was in fact 7% (non-significantly) higher (De Schryver *et al* 1969). The reports of De Schryver *et al* are also at variance with that of Hecht *et al* (1973) who reported that with similar low intensity exercise (running speed 6 m/min) but daily period of running increasing up to 7 h/day there was as in the studies of De Schryver *et al* an absence of cardiac hypertrophy but there was no significant change in cardiac NA concentration; indeed it tended to be higher (non-significantly) in the trained rats. It seems somewhat unlikely that strain differences could explain the discrepancy in results between De Schryver and co-workers (who used Wistar rats) and other workers in the field, since in eight other publications using three different strains of albino rats (unselected albino rats, Charles River C.D. rats Sprague-Dawley rats) as well as guinea pigs and various training schedules varying from mild to heavy there is no other observation even of non-significant decrease in total NA content of the heart of chronically exercised animals (papers I, II III V Secor, E this chapter Ortmann and Spöstrand, 1971 Hecht *et al* 1973 Leon *et al* 1975).

Furthermore there are several obscure aspects of the reports from De Schryver *et al*. Firstly they state in all their publications that "the growth curves were the same in controls and trained animals as judged from their published data; however the growth curve must be highly abnormal since the reported weight increase in their male rats during an experiment lasting 3 months is about 90 g, with range of 40–110 g (De Schryver *et al* 1969). A similar very low increase in body weight, averaging 80 g during the three month experimental period can be deduced from their previous data (De Schryver *et al* 1967) if one uses the subsequently published heart ratios to calculate the final body weight which is not given in that publication. Young male rats normally grow very rapidly; thus for example rats of the Kyoto-Wistar strain and of ordinary Wistar strain with the same initial body weight as the ones used by De Schryver and associates would be expected to gain about 165 g and 250 g respectively over 12 week period (see Ozaki *et al* 1972 and Takeda and Keshu 1972). Similarly young rats of our strain of Sprague-Dawley rats would gain about 270 g over 3 months (paper VI) as would Charles

River C.D. rats (Leon *et al.* 1976). Both these strains and the Wistar rats of Ozaki *et al.* (1972) reach body weights of the order of 400–490 g at the same age as that when the rats of De Schryver *et al.* are reported to weigh about 240 g. Thus, either the growth of the rats employed in their studies is severely stunted by some aspect of their experimental conditions or their experiments were not designed in proper longitudinal fashion. If one uses the data on heart weight and heart ratio given by De Schryver and Mertens–Brythagen (1972) to calculate the mean body weight of the different experimental groups it is found that for example the mean body weight of the 600 m/h group killed after one week of training is 268 g whereas the mean body weight of that killed after 8 weeks of training at 600 m/h is 232 g! Male rats continue to grow throughout life and it is inconceivable that a young male rat maintained with a reasonably adequate food supply should fail to gain weight, and instead lose weight, over a period of 7 weeks. Thus, one is forced to conclude that a false impression is given in their Methods description of a “longitudinal design of the experiment (e.g. “at the beginning of the experiment the rats were divided into several groups” De Schryver *et al.* 1972) and that in fact some other experimental design has been used, resulting in all experimental groups being of roughly the same body weight at death regardless of the duration of training. This is borne out by the fact that in a study involving 20 different experimental groups there is only one single group of untrained animals used as controls for experiments spanning from periods of one to nine weeks training on the motor-driven drum. Such an experimental design would mean that none of the exercised groups were comparable to each other since they would all have started the exercise at a different age. All these aspects of the work of De Schryver and his associates would suggest that they have not used adequate control groups to exclude that their reports of decreased NA content and concentration in the hearts of their “exercised” rats is not in fact an artifact derived from their experimental design.

In summary therefore it would seem that, disregarding the work of De Schryver and associates there is no discrepancy between the findings of different workers on the NA content and concentration in the heart of chronically exercised animals that cannot be accounted for by the use of different training parameters.

*Long term effects of exercise.* What happens to the sympathetic innervation of the heart in a chronically exercised rat that is then allowed to rest for a prolonged period? In paper III it was found that although there was still significant cardiac hypertrophy (expressed as heart ratio) the cardiac total NA content and NA concentration were no longer significantly different from untrained controls 2 months after the last exercise period. The lack of statistical significance was due to large inter-individual variation in the two groups, both the NA content (+28%) and the NA concentration (+18%) of the heart were in fact, considerably higher in the exercised group than in the controls. Inter-individual variation in body and organ size increases with age in the rat and if the very highest and the very lowest observations in both of the two groups of rats studied 6 months after cessation of exercise are excluded it is then found that total NA content (controls  $1.16 \pm 0.12 \mu\text{g NA}$ , exercised  $1.50 \pm 0.07 \mu\text{g NA}$ ,  $p < 0.05$ ) is probably significantly higher in the exercised rat. As regards the NA concentration in the heart (controls  $0.74 \pm 0.08 \mu\text{g NA/g}$ , exercised  $0.93 \pm 0.04 \mu\text{g NA/g}$ ,  $0.05 < p < 0.1$ ) the remaining difference only just falls short of statistical significance. This is in some contrast to the report of Unger *et al.* (1973) that in the female rats there did not seem to be an increased density of noradrenergic terminals in the heart two months after cessation of training. However probably due to different experimental conditions, as discussed earlier (Section B) they do not see persisting cardiac hypertrophy either which is seen in our male rats. Assuming that at least part of the increased cardiac NA stores in trained rats is due to formation of new terminal nerve fibres accompanying

varicose reformation as discussed above, it seems rather unlikely that sympathetic varicose fibres having once developed should degenerate on cessation of physical exercise in particular since the increased capillary density induced by physical exercise persists for at least two months after the regular exercise is terminated (Ljungqvist and Unger 1972). A continued presence of an increased number of varicose nerve fibres would not of course mean a continued presence of an increased total NA content unless the NA content of each varicose fibre was maintained. In the absence of continued periods of swimming there would be no further stimulus, through increased nerve impulse flow to induction of synthesis of higher than normal amounts of tyrosine hydroxylase and dopamine  $\beta$ -hydroxylase. Thus one might speculate that there may be an intrinsic mechanism operating in the neuron which prevents the transmitter concentration in each varicose nerve fibre as distinct from each neuron, from falling below certain minimum, and that this minimum is not greatly different from that seen in normal control rats.

#### *Submandibular gland*

The total content and concentration of NA in the submandibular salivary glands were not significantly influenced by chronic physical exercise as might be expected since this organ is not directly involved in the cardiovascular adjustments to physical work.

#### *Spleen*

The results on total content of NA in the spleen in papers III and V are somewhat conflicting in that whereas in paper III there is a significant increase in total NA content (28%) there was only non-significant increases of 3% in paper V. The reasons for this discrepancy are not clear: it seems very unlikely that the more variable recovery of the assay procedure in paper V could on its own account for such a diminution of differential. Possibly a time factor could be involved since the rats in paper III had been swim-trained for 14 weeks, with the daily swimming period increased to 2 h/day and the rats in paper V on the other hand had been trained for 17 weeks with the daily exercise period increased to 2½ h/day. Could it be that the development in trained rats of increased sensitivity to the vasoconstrictor actions of NA (see Section G) so diminishes the demand for increased sympathetic nervous activity in the spleen during exercise that the stimulus for increased storage of NA in splenic nerves disappears? It is noteworthy that the activity of sympathetic nerves in the spleen of the trained rats in paper V is in fact markedly decreased during resting conditions, which would tend to lend some support to the above interpretation.

There is little doubt, however, that the concentration of NA in the spleen remains elevated, since if the data on NA concentration in the spleen from paper V are pooled with data from other rats out of the same experimental groups used for other experiments one finds a value of  $0.94 \pm 0.06 \mu\text{g NA/g}$  for control rats ( $n = 15$ ) and  $1.14 \pm 0.06 \mu\text{g NA/g}$  for trained rats ( $n = 15$ ,  $p < 0.02$ ). It can be argued, though, that the NA concentration in the spleen in this case is rather irrelevant since it probably results from decrease in the amount of lymphoid tissue in the spleen (see Section B).



## SECTION D URINARY EXCRETION OF CATECHOLAMINES AND VANILLYL MANDELIC ACID

(Papers I, III, VI and unpublished data)

### RESULTS

#### *At rest*

In paper I where the trained rats were rested for 24 h before the collection of urine, no difference was found between trained and untrained rats as regards the excretion of NA and adrenaline in the urine at rest. In paper III however, where the collection of urine was preceded by 48 h rest in both groups (the control rats having had their one and only bout of swimming for the purpose of urine collection 48 h earlier) the urinary excretion of NA in trained animals was significantly lower than that of controls, being 87% of the control value ( $p < 0.001$ ) while the excretion of adrenaline in the urine was not significantly different from that of controls.

#### *After exercise*

In untrained rats a 2 h period of swimming was found significantly to increase the urinary excretion of both NA (II +124%,  $p < 0.001$  III +44%,  $p < 0.001$ ) and adrenaline (II +300%,  $p < 0.001$  III 36%,  $p < 0.05$ ) during the 22 h following the swimming session. In the trained groups the excretion of NA in the urine was also significantly elevated after exercise but the rise was smaller both in absolute terms ( $\mu\text{g}/22 \text{ h}$ ) and as a proportion of the resting value. Thus in trained rats the urinary excretion of NA after exercise was only 84% (I) to 85% (I) to 85% (I) of that of the respective control groups. The rise in the excretion of adrenaline in the urine after exercise was also significantly smaller in trained than in untrained rats in both studies: in paper I the net increase in adrenaline excreted was only 33% of that seen in control rats, while in paper III the rise in adrenaline excretion after exercise was not even significant in the trained group. The proportion of total catecholamines excreted in the urine that was constituted by NA was however not significantly changed after exercise in either trained or untrained rats (paper VI).

#### *Excretion of vanillyl mandelic acid*

During resting conditions excretion of vanillyl mandelic acid (VMA) in the urine was lower in the trained group, being  $23.5 \pm 1.2 \mu\text{g VMA}/22 \text{ h}$  ( $n = 12$ ) as compared with  $27.5 \pm 2.0$  in the control rats ( $n = 12$ )  $p < 0.05$  (previously unpublished data).

#### *Several months after cessation of exercise (paper III)*

6 months after cessation of exercise there is no significant difference between the trained rats and their age-matched control group in the excretion of NA and adrenaline in the urine at rest and during cold stress. After exercise however the trained rats still excrete less NA (-16%) than untrained rats do ( $p < 0.05$ ). Unlike the situation in fully trained rats the excretion of adrenaline in the urine after exercise is the same in trained rats as that found in controls. The persistence of trained rats of a lower degree of overall sympathetic activity during exercise as monitored by the urinary excretion of NA, is also verified when the net increase in the amount of NA excreted by individual rats was

determined in paired observations (controls:  $0.78 \pm 0.10 \mu\text{g NA}/22 \text{ h}$  10, ex-trained:  $0.41 \pm 0.07 \mu\text{g NA}/22 \text{ h}$  11  $p < 0.02$ )

## DISCUSSION

Our finding that exercising rats by swimming leads to an increased excretion of NA and A in the urine confirms earlier findings on humans (see Historical Introduction and review by Euler 1974). Since in contrast to the above mentioned studies, we were not able to collect urine formed during the performance of the exercise, we studied the excretion of CA in the urine soon after the peak levels of catecholamines in plasma must have occurred and during the subsequent 22 h period of recovery and rest.

One cannot be completely certain that all the rats had empty bladders after swimming (see Chapter 2) but, assuming that most of the urine formed during swimming was lost in the swimming bath, it is likely that the increase in urinary CA excretion evoked by exercise was greater than that actually measured. A second factor which makes it difficult to compare the present results with those obtained in humans is the fact that the rate of CA excretion was measured over a 22 h period while in humans it can be measured over much shorter intervals. Kerk (1956) suggested that in man the urinary excretion of catecholamines returns to normal after physical exertion in about one hour; this is probably not quite the case in rats, since our results both after exercise and cold exposure suggests that it takes more than 24 h for a rat to reach truly basal excretion values of NA. After these two types of physiological stress (see later in Discussion and Chapter 4). However, in order to make a very rough comparison between the species, one can allocate the net increase in catecholamine excretion occurring after exercise in rats to the 2 h immediately following the swimming period, and compare that rate of catecholamine excretion ( $\mu\text{g/h}$ ) with the normal resting excretion rate for each group, expressed as  $\mu\text{g/h}$  (this gives at least a very approximate idea of the severity of the work load although, due to the loss of urine during swimming, it would tend to underestimate the increase in catecholamine excretion in the rats). Using the data from paper III, it is then calculated that control rats have about six-fold increases in urinary excretion of NA, and about five-fold increase in adrenaline excretion, immediately after exercise as compared with excretion at rest, while the corresponding values for trained rats are five-fold increase in NA, and three-fold increase in adrenaline, excretion in the urine. This magnitude of increase suggests that the work load involved in swimming is considerable, since in humans it needed a work load of more than 80% of the individual maximal oxygen uptake to increase the arterial plasma concentration of NA more than five-fold (Haggenfeld *et al.* 1970) and since it is known that although supramaximal work in humans can increase plasma NA concentration 80-fold already 5 min after work the plasma concentration of NA has come down to 10–12 times the resting value (Johnson *et al.* 1974).

### Excretion of adrenaline

There was no difference between trained and untrained rats in resting excretion of adrenaline (papers II and III). The absolute values of resting excretion of adrenaline, however, were considerably lower in paper II than in paper III although the amounts excreted after exercise were about the same in the two studies. The excretion of CA in the urine is known to vary according to the time of year (Ladue, 1961) and this is probably the explanation for the difference in resting CA excretion in the two studies. It is noteworthy that when the resting excretion of adrenaline is comparatively high as in paper III, there is no further statistically significant increase in the urinary excretion of adrenaline after exercise in the trained group, whereas when the resting excretion is comparatively low as in paper II, exercise increased the excretion of adrenaline even in trained rats.

In human subjects, who are motivated and therefore probably not too emotionally stressed by an exercise session physical exertion increases the plasma levels, as well as the urinary excretion of both NA and adrenaline (for references see Chapter II). Emotional stress, on the other hand causes less mainly

In the urinary excretion of adrenaline both in humans (see review by Frankenhauser 1971) and in rats (Kvetnensky and Mikulel, 1970). Therefore the finding that trained rats exhibit a much smaller rise in urinary excretion of adrenaline after exercise than control rats do while their resting adrenaline excretion remains the same as that of controls, could at least partly be due to an adaptation to the emotional stress of swimming. Such an adaptation is however unlikely to be the sole factor involved, since intermittently exercised rats, well adapted to the stress of swimming, still excrete more adrenaline in their urine after exercise than daily exercised rats do, although they excrete significantly less than control rats do (see Section E). This points to the existence of adaptive changes in adrenaline secretion induced by the physical exertion itself. Such changes are not likely to be predominantly secondary to changes in tissue sensitivity to the "calorigenic" actions of adrenaline since the adrenaline excretion both during rest and during cold stress, a potent stimulus for adrenaline secretion, is the same in trained rats as in controls (see also Chapter 4).

It also deserves to be pointed out that after exercise there was a parallel increase in urinary excretion of NA and adrenaline even in the untrained rats so that the proportion of NA was unchanged from rest ( $87.5 \pm 0.8\%$ ) compared with after exercise ( $88.3 \pm 1.0\%$ ) (paper VII). Thus it seems likely that physical exertion, rather than emotional stress, was the most prominent factor causing the observed activation of the sympatho-adrenal system during swimming even in the untrained rats unaccustomed to water.

#### *Excretion of noradrenaline*

The discrepancy between paper II and III on the question of whether or not trained rats excrete less NA in the urine than controls do during rest as well as after exercise, is probably due to the different time schedule used. It is likely that it takes more than 24 h after exercise before truly basal resting conditions are achieved, since no difference in excretion was found between trained rats and controls 24 h to 48 h after the last period of exercise whereas from 48 h to 72 h after exercise the trained rats excreted less NA than control rats by the same proportion (i.e. 67% of the control value) as that seen immediately after exercise in both studies (66 to 64% of control value). Thus the results suggest that chronically exercised animals require a lower degree of overall sympathetic tone in order to maintain homeostasis while at rest as well as during exercise. This is true for human subjects as well both at rest and during and after exercise (Johnson *et al.* 1974). A lowered sympathetic tone, as reflected in decreased half life of tissue stores of NA, has been shown to exist both in the heart and in the spleen of trained rats (see Section F). Since the nerves innervating the heart and spleen and the adrenal medullary secretion give rise to only a minor fraction of the NA excreted in the urine at rest whereas the major fraction arises from nerve terminals in the vasculature (Bigelow *et al.* 1969; Spector *et al.* 1972) it is unlikely that these findings on their own could account for the observed diminution of urinary excretion of NA in trained rats. However, the increase in the sensitivity to the vasoconstrictor actions of NA found in the vascular bed of trained rats (see Section G) could well account for the observed difference in NA excretion in the urine as trained rats are known to maintain normal blood pressure (Criss and Aldinger 1967; Ljungqvist and Unger 1972).

The possible occurrence in trained rats of increased sensitivity to calorigenic actions of NA (see Chapter 4) might also be a contributory factor.

#### *Vanillyl mandelic acid excretion*

An increase in the rate of metabolic degradation of released catecholamines in trained rats is a hypothetical mechanism whereby a decreased excretion of NA and adrenaline in the urine could be explained. In order to exclude this possibility the urinary excretion of one of the major metabolites,

VMA, was studied. The finding that trained rats excreted significantly less VMA in the urine at rest than the control rats did, is evidence that there is in fact no increased metabolism of catecholamines in the trained rats. Furthermore there was no significant difference in the relative proportion of total CA excretion to VMA excretion in the two groups (unpublished observation). Thus, the lower CA excretion in trained rats is not due to increased metabolism but must reflect an overall decrease in sympatho-adrenal secretory activity.

#### *After cessation of exercise*

6 months after cessation of exercise the overall sympathetic activity as monitored by urinary NA excretion, observed in trained rats is no longer significantly different from control values during rest (92% of control excretion) whereas the urinary excretion of NA after exercise was still significantly lower (84% of control value). The persistence in ex-trained rats of lower urinary excretion of NA after exercise could at least partly be due to the persistence of cardiac hypertrophy in these rats (see Section B) leading perhaps to less sympathetic nervous activity in the hearts of these animals. Although the NA overflowing from the heart is probably a minor fraction of the NA excreted in the urine at rest (see above) it probably constitutes a more significant fraction of the NA excreted in the urine after exercise since the NA turnover in the heart of these rats increases 10–17 fold during swimming (see Section F). On the other hand, the unchanged resting excretion of NA suggests that the increased sensitivity to the vasoconstrictor actions of NA seen in trained rats (see Section G) is not permanent but disappears after discontinuation of the regular exercise as is also the case with the increased tissue sensitivity to calorigenic actions of NA in cold-acclimated rats retransferred to a warm environment (Lienky *et al.* 1967).

## SECTION E EFFECTS OF INTERMITTENT EXERCISE

The effects of intermittent as opposed to daily exercise were studied in a previously unpublished experiment.

### MATERIALS AND METHODS

Male Sprague-Dawley rats, initially weighting about 170 g were divided in three groups, one served as controls, one was submitted to daily swimming exercise according to the schedule published in paper II with the daily swimming time increased stepwise from 1 to 2 h/day. The third group was submitted to swim training three times a week only on alternate week days, but was given the same increase in daily swimming time at the same time after the start of the experiment, as the daily exercised group. The total duration of swim training was 18 weeks and the urine collections were performed during the 14th week. Urine was collected during 22 h period firstly after 24 h of rest and secondly immediately after 2 h swimming period. Urine collection and CA extraction and determination in organs were performed as described in Chapter 2. Cardiac NA content was assayed according to Cheng (1964) and CA content in adrenal glands and urine according to Euler and Lohajko (1961).

### RESULTS

The results are summarized in Table 1 and Fig. 1. Whereas intermittent exercise in contrast to daily exercise has no effect on body weight, it does on the other hand produce the same degree of,

Table 1 Comparison of the effect of daily and intermittent exercise on organ weight and organ catecholamine content.

Percentage figures given within parenthesis show the increase in catecholamine content as percentage of control value.

Symbols: different from control  $p < 0.05$     different from control  $p < 0.01$     different from control  $p < 0.001$     ■ different from the daily exercised group  $p < 0.01$     c, different from the daily exercised group  $p < 0.001$

	Controls	Intermittent	Trained
Initial BW (g)	173 $\pm$ 2	173 $\pm$ 2	175 $\pm$ 1
Final BW (g)	427 $\pm$ 4	438 $\pm$ 4 <sup>a</sup>	407 $\pm$ 4
Heart wt (g)	1.28 $\pm$ 0.02	1.39 $\pm$ 0.02 <sup>a</sup>	1.37 $\pm$ 0.02 <sup>a</sup>
Heart ratio g/100g BW	0.298 $\pm$ 0.003	0.311 $\pm$ 0.003 <sup>a</sup> <sup>c</sup>	0.334 $\pm$ 0.005
Adrenal wt (mg)	41 $\pm$ 1	46 $\pm$ 1	45 $\pm$ 1
<u>Heart</u>			
NA $\mu$ g	0.83 $\pm$ 0.03	1.11 $\pm$ 0.04 (+ 19%)	1.16 $\pm$ 0.04 (+ 25%)
NA $\mu$ g/g	0.73 $\pm$ 0.03	0.80 $\pm$ 0.03 (+ 10%)	0.85 $\pm$ 0.03 <sup>a</sup> (+ 16%)
<u>Adrenals</u>			
NA $\mu$ g	6.26 $\pm$ 0.58	7.94 $\pm$ 0.36 <sup>a, b</sup> (+ 27%)	12.14 $\pm$ 1.03 (+ 94%)
Adrenaline $\mu$ g	35.6 $\pm$ 1.7	41.6 $\pm$ 0.7 (+ 17%)	42.8 $\pm$ 2.4 (+ 20%)
% NA of CA	16 $\pm$ 1	16 $\pm$ 1	22 $\pm$ 1 (+ 47%)

cardiac hypertrophy as daily exercise does, expressed as increase in absolute heart weight. Hypertrophy of the adrenal glands also occurs in intermittently exercised rats and is of the same magnitude as that seen after daily exercise. The effects on the CA content of the heart and adrenal glands, on the other hand, are not so marked as those which occur after daily exercise. As in the case in daily exercised rats the urinary excretion of both adrenaline and NA after 2 h period of exercise was significantly lower in intermittently exercised rats as compared with controls. When intermittently exercised rats are compared, on the other hand with the group that was exercised daily there is no significant difference between the amounts of NA excreted in the urine after exercise whereas the amount of adrenaline excreted is even lower in daily exercised rats than it is in the intermittently exercised group ( $p < 0.05$ ).

## DISCUSSION

The finding that intermittently exercised rats do not exhibit the decreased body weight found in animals that were exercised daily (for references see Chapter 1 and Section B of this Chapter) suggests that the alternate day of rest ensures enough of a compensatory increase in food intake on the day of rest to make up for the relative hypophagia on the day of the exercise session (for references see Discussion in Section B) and for the increase in energy expenditure during the physical exertion.

The observation that intermittently exercised rats display the same degree of cardiac hypertrophy (+8%) as daily exercised rats (+7%) suggests that it is the intensity and duration of exercise sessions, rather than the accumulated total hours of exercise, that determines the degree of cardiac hypertrophy obtained after physical training (see also Discussion in Section B). My findings on body weight and heart weight are seemingly at variance with the results of Leon and Bloor (1968) and Bloor and Leon (1970) who reported that intermittent exercise leads to cardiac hypertrophy in very young rats only not in adult rats, but that it always leads to body weights significantly below control values. This discrepancy is probably due to different experimental procedures. Thus the absence of cardiac hypertrophy in intermittently exercised rats in their experiment is probably due to less severe training schedules, one h of swimming twice weekly as compared to the 2 h of swimming three times a week that the intermittently trained group was subjected to in this experiment. Furthermore Leon and Bloor swim their rats in water at temperature of 28–32°C as compared to the water temperature of 24–36°C used in our swim studies, and thereby add an appreciable element of cold stress in their experiment. This could perhaps explain why they see a reduction in growth rate in their intermittently exercised animals as well since cold exposure can lead to decreased weight gain (Schwabe *et al.* 1938; Heroux and Hart 1964).

The fact that the heart ratios in the intermittently exercised rats were significantly lower than those of the trained rats, is only a reflection of their higher body weight (see Discussion in Section B).

Intermittent training also does not differ from daily exercise in the ability to produce adrenal hypertrophy which again suggests, just as in the heart that as long as the exercise sessions are fairly frequent it is more the work load in the individual session rather than the accumulated hours of work that determines the degree of adaptive adrenal cortical hypertrophy.

On the other hand, intermittent exercise does not produce as much increase in the NA content of the adrenal gland as daily exercise does, and although the NA-content in the heart is not significantly lower than after daily exercise the percentage increase in NA-concentration compared with controls is nevertheless smaller +10% as compared to +18%. Thus it appears that the mechanisms responsible for the increase in heart and adrenal CA content seen in trained rats, namely enzyme induction and in the case of the heart perhaps also growth of new terminals (see Section C) need daily stimulus to produce maximal increases in transmitter content. This fits with the finding of Leon and Bloor (1968) that intermittent exercise caused rise in capillary/fibre ratio that was only about 60% of the rise seen in rats that were exercised daily.

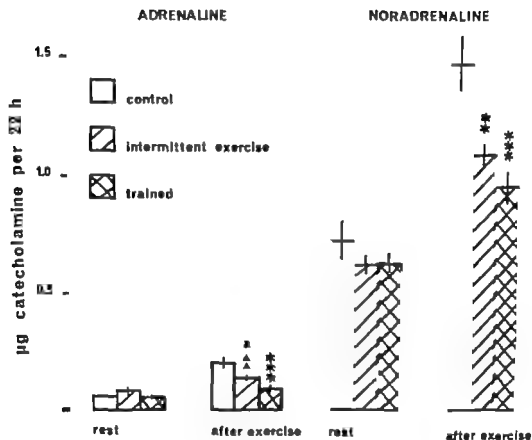


Fig. 1 Comparison of the effect of daily and intermittent training on the excretion of catecholamines in the urine

The values are given as  $\mu\text{g}$  catecholamine/22h: mean  $\pm$  S.E.

Symbols: open bars: control values; striped bars: values from intermittently exercised rats; cross hatched bars: values from rats subjected to daily exercise. \* different from control  $p < 0.01$ ; \*\* different from control  $p < 0.001$ ; AA different from control  $p < 0.02$ ; different from the daily exercise group  $p < 0.05$ .

The "resting" urine collections were made 24 h after the last period of exercise. In view of our later finding (paper III) that at least 48 h of rest is probably necessary to reach a truly basal level of sympathetic nervous activity and thereby minimal excretion of NA in the urine, significant difference between intermittently exercised rats and control rats in the resting urinary excretion of NA might therefore have been masked.

However, the finding that both the trained and intermittently exercised groups excrete significantly less NA after exercise than control rats do (see Fig. 1) suggests that intermittently exercised rats may share the decrease in overall sympathetic nervous activity found in daily exercised rats both at rest and after exercise (see Sections D and F) possibly as a result of increases in peripheral sensitivity to the vasoconstrictor action of NA (see Section G) and perhaps mediated via an increase in the inhibitory activity of noradrenergic neurons in the brain as discussed in Chapters 5 and 7.

Intermittently exercised rats can be expected to have adapted to the emotional stress of the severe training to the same degree as rats trained by daily exercise. However, the amount of adrenal neurtic acid in the urine of intermittently exercised rats after exercise was significantly higher than that of daily exercised rats ( $p < 0.05$ ) although it was still significantly lower than that of controls ( $p < 0.02$ ). This observation suggests that adaptation to the emotional stress of swimming is not the only factor contributing to the diminution of exercise-induced rise in urinary excretion of adrenaline seen in trained rats, but that there is a further factor, presumably an adaptive response to repeated physical exertion itself. Furthermore, it appears that the latter adaptive response is only fully expressed in animals subjected to daily exercise sessions, as opposed to sessions on alternate days. This adaptation could involve cardiovascular and/or "metabolic" e.g. substrate mobilizing, actions of catecholamines and it is noteworthy in this context that intermittently exercised rats do not achieve the significant increase in the proportion of adrenal CA content that is comprised by NA that is found in daily exercised rats.

## SECTION F. TURNOVER OF CATECHOLAMINES IN VARIOUS ORGANS

(Papers II, III and unpublished observations)

During steady state conditions the CA content in the neuron is maintained because the rates of synthesis and reuptake are equal to the rates of release and intraneuronal degradation. Thus turnover of the transmitter is equivalent to release plus intraneuronal degradation less reuptake and equals the rate of synthesis during steady state conditions. The rate of NA turnover is proportional to the amount of nervous activity in the peripheral sympathetic neuron and turnover is virtually abolished by decentralization (Olivero and Sjoerdsma 1965; Alouf and Warner 1966; Gordon *et al.* 1966<sup>a</sup>; Bhargava, 1967; Sachdev *et al.* 1968; Sweden, 1970). Since transmitter concentrations in adrenergic neurons are acutely altered only during experimental conditions that lead to very marked and/or prolonged increases in nervous activity (see Section C for references) it is essential to study the turnover of NA, rather than the endogenous levels, in order to detect more moderate alterations in nervous activity. Various methods have been designed to study turnover rate but undoubtedly the most physiological ones are those that avoid any pharmacological manipulations by achieving radioactive labelling of transmitter stores, either by administration of labelled NA or of labelled precursor. The turnover rate can then be estimated by applying steady state kinetics to the disappearance of the labelled transmitter (see Montanari *et al.* 1963).

After administration of  $^3\text{H}$ -NA the disappearance of the labelled substance follows an exponential time course from 15 min onwards in the rat heart (Iversen *et al.* 1971). The rate of



of labelled DA and of NA, formed from labelled tyrosine, has been shown to be exponential in the rat brain from 2 h or earlier after injection of the labelled precursor (Persson 1969; Nybeck, 1971) and in the rat heart from 1 h onwards (Persson, 1969).

In order to obtain an estimate of sympathetic nervous activity in organs of chronically exercised rats the turnover of NA has been studied using both exogenous  $1\text{-}^3\text{H}$ -NA and labelling of endogenous NA by  $L\text{-}^{14}\text{C}$ -tyrosine.

## RESULTS

The estimated half-lives of the transmitter stores in various organs are given in Table 2 and Fig. 2, based on some previously unpublished results as well as data from papers II and III. The data from paper II are converted from specific activity in  $\mu\text{Ci}/\mu\text{g}$  NA, to the total  $\mu\text{Ci}$  of  $^3\text{H}$ -NA per organ since the endogenous NA levels were altered during one of the experiments.

### Brain

The turnover rates of DA are the same in trained rats and control rats both at rest and after exercise and the turnover rate is not changed by exercise in either group. The half-lives of NA are also the same in both groups, but since the trained rats have significantly more NA in their brains (see Section C) the actual amount of NA turning over per unit of time is higher in trained rats. Using the formula of Brodie *et al.* (1966) (total turnover equals steady state concentration times the rate constant [ $\log_e$  base] for disappearance) and correcting the steady state concentration for the recovery assay method, the minimum turnover rate of  $0.088 \mu\text{g NA/g/h}$  is calculated for trained rats, compared with  $0.070 \mu\text{g NA/g/h}$  for the controls, representing an increase of 26%. (Due to a misprint in Table 1 in Paper V where the NA concentration in the brains of trained rats was given as  $0.37 \mu\text{g/g}$ , rather than the correct figure of  $0.35 \mu\text{g/g}$ , an erroneous turnover figure of  $0.093 \mu\text{g/g/h}$  for the trained rats was unfortunately given in paper V.)

There appears to be an increase in NA-turnover in the brain while the rats are swimming, compared with when they are at rest, both in controls (+42%) and in trained rats (+31%); however, only in the trained group is the slope of the regression line of  $\mu\text{Ci}$  of  $^{14}\text{C}$ -NA versus time significantly smaller ( $p < 0.02$ ) at rest than during swimming (previously unpublished; it must be emphasized that the turnover rates of CA in the brains of swimming rats should only be compared with the resting values because in this experiment the trained rats, that were swimming in separate tanks from the control rats, displayed much more active swimming than control rats did and thus these groups are not strictly comparable with each other).

### Heart

The half-life of NA in the hearts of trained rats is significantly longer than in the hearts of control rats. This was shown independently by using either exogenous  $1\text{-}^3\text{H}$ -NA (paper II) or  $L\text{-}^{14}\text{C}$ -tyrosine (paper V) for labelling of transmitter stores. Using data from paper II the total turnover of the transmitter store can be estimated to be  $0.041 \mu\text{g NA/g heart/h}$  in the controls but only  $0.010 \mu\text{g NA/g heart/h}$  in the trained rats. In fact in both studies the slopes of the regression lines of labelled NA versus time are not significantly different from zero in the trained groups and thus one can say that there is no significant turnover of transmitter detectable over 12 h period, in the heart of resting trained rats. During swimming there is a marked increase in cardiac NA turnover in both groups, but the fractional turnover rate is still significantly lower in trained rats ( $p < 0.01$ ) with a  $t_{1/2}$  of 2.5 h compared with 1.4 h in the controls. Furthermore, the rate of NA turnover is significantly lower in the hearts of

Table 2. The turnover rate of dopamine and noradrenaline in brain, heart and spleen of trained and untrained rats, at rest and during swimming.

Symbols:  $t_{1/2}$  = half-life;  $k$  = rate constant; S.D. = standard deviation

	Slope (k) ± S.D	t <sub>1/2</sub> (h)	k ≠ 0	Method
BRAIN NA				
<u>Rest:</u>				
Controls	-0.064 ± 0.012	5.5	p < 0.01	L- <sup>14</sup> C-tyrosine
Trained	-0.065 ± 0.006	5.5	p < 0.001	
<u>Swimming:</u>				
Controls	-0.077 ± 0.013	3.9	p < 0.001	L- <sup>14</sup> C-tyrosine
Trained	-0.072 ± 0.012	4.2	p < 0.001	
BRAIN DA				
<u>Rest:</u>				
Controls	-0.082 ± 0.006	3.7	p < 0.001	L- <sup>14</sup> C-tyrosine
Trained	-0.080 ± 0.006	3.3	p < 0.001	
<u>Swimming:</u>				
Controls	-0.093 ± 0.014	3.2	p < 0.001	L- <sup>14</sup> C-tyrosine
Trained	-0.092 ± 0.012	3.3	p < 0.001	
HEART NA:				
<u>Rest:</u>				
Controls	-0.016 ± 0.004	18.6	p < 0.01 n.s.	L- <sup>3</sup> H-NA
Trained	-0.003 ± 0.005	104.0		
Controls	-0.037 ± 0.017	8.0	p < 0.005 n.s.	L- <sup>14</sup> C-tyrosine
Trained	-0.004 ± 0.012	80.0		
<u>Swimming:</u>				
Controls	-0.214 ± 0.063	1.4	p < 0.01 p < 0.005	L- <sup>3</sup> H-NA
Trained	-0.119 ± 0.018	2.6		
SPLEEN NA				
<u>Rest:</u>				
Controls	-0.064 ± 0.020	5.5	p < 0.001 n.s.	L- <sup>14</sup> C-tyrosine
Trained	+0.00006 ± 0.017	∞		

Note that all the numerical values of  $k$  in this table have been derived from regression lines where the  $y$ -values have been expressed in  $\log_e$ , thus those values originating from paper V have been converted from  $\log_{10}$ -based values to  $\log_e$ -based values.

trained rats compared with controls also during intermittent swim exercise (see Fig. 2 data from paper II)

### Spleen

In this organ as well the fractional turnover rate of NA was significantly lower in trained rats than in control rats. The slope of the disappearance of the radioactively labelled NA versus time was not significantly different from zero in the spleen of the trained rats, and the estimated half-life was "infinite".

## DISCUSSION

### Turnover of DA and NA in the brain

The calculated half-lives of DA and NA reported in paper V are in good agreement with previously published half-lives estimated by different methods (Iversen and Glowinski 1966; Taylor and Laverly 1969; Parson 1969 and Nybeck, 1971). The half-lives of both NA and DA in the brain, as estimated by disappearance of  $^{14}\text{C}$ -CA after  $^{14}\text{C}$ -tyrosine administration, are not changed by chronic exercise. However, since both the concentration and the total content of NA, but not those of DA, are significantly increased in brains of the chronically exercised rats, the total amount of NA turning over per unit time is increased in the brains of the trained animals at rest. This finding is very unlikely to be due to any persisting effect of the acute physical stress of the last swimming period since the turnover study using  $^{14}\text{C}$ -tyrosine was preceded by 48 h of rest and since the turnover rates of NA in the two peripheral organs studied were in contrast to that in the brain, markedly lower in trained than in control rats. Thus the observed increase in total NA turnover in the brain of trained rats is unlikely to be an experimental artefact.

The increased NA content in the brain of chronically exercised rats might be an adaptive response to daily bouts of increased nervous activity in noradrenergic neurons, as discussed in Section C. It is well known that during forced running of untrained rats there is an increased turnover of NA in whole brain and brain stem (Gordon *et al.* 1965<sup>b</sup>) as well as hypothalamus (Stone 1971, 1973). Heat has been found to increase the turnover of NA in whole brain (Corrodi *et al.* 1967) as well as the hypothalamus (Simmonds, 1969) and forced running can easily induce hyperthermia. However, as shown in Table 2, an increase in turnover rate of NA in whole brain occurs during swimming just as it does with forced running. Since this increase occurs in trained rats which are emotionally adapted to swimming and is of the same magnitude as in controls, it appears that the increase in turnover rate of NA in the brain may be a response to muscular work rather than to more non-specific factors such as emotional stress. In this context the possibility must also be considered that, during exercise there is increased activity in noradrenergic pathways that might participate in the hypothalamic regulation of endocrine functions since it is known that both ACTH (Frenkl *et al.* 1968, 1969) and growth hormone (Hunter *et al.* 1965; Sutton *et al.* 1969; Hartley *et al.* 1972) levels in plasma are elevated by exercise. In fact changes in the turnover of NA in noradrenergic neurons in the hypothalamus are perhaps more likely to account for the observed increase in total brain turnover of NA than for example noradrenergic neurons in the lower brain stem. This is because the activity of some of the latter pathways is assumed to stand in an inverse relationship to peripheral sympathetic nervous activity (see Chapters 5 and 7) which, of course, is markedly increased during exercise.

It is noteworthy that 3 h of moderate swimming activity does not increase the turnover rate of DA in the brain either in controls or in chronically exercised rats (Table 2). Bins and Aulio (1971) have reported that swimming in water at 37°C leads to increased levels of homovanillic acid (HVA)

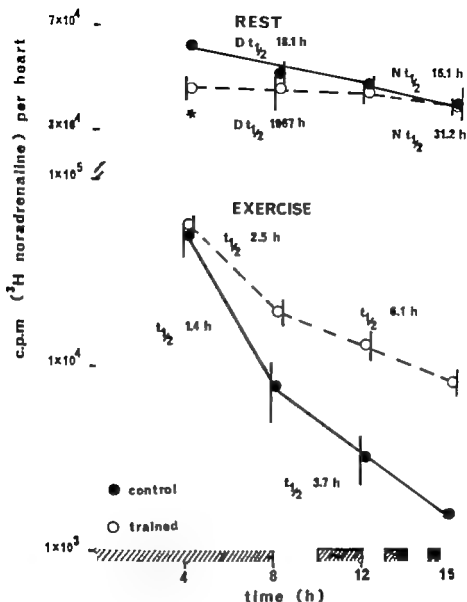


Fig. 2. The effect of chronic exercise on cardiac turnover of noradrenaline at rest and during exercise. Symbols as in figure.

The values given are means  $\pm$  S.E. note that the logarithmic scales on the y-axis are different in the two parts of the figure.  $Dt_{1/2}$  denotes half-life during day time (the period 4h–12h)  $Nt_{1/2}$  the half-life during night time (the period 12h–16h) denotes different from control  $p < 0.05$ . Data from paper III but expressed as c.p.m. ( $^3\text{H}$ -NA) per heart rather than as previously c.p.m./ $\mu\text{g}$  NA. The shaded areas along the x-axis shows the periods during which the two groups of rats were subjected to swimming for the determination of cardiac NA turnover during continuous (0–8h) and intermittent (8–16h) exercise.

the brain of mice but this need not be at variance with the above findings. DA turnover since Hutchins *et al.* (1978) have shown that the proportion of DA metabolites that is constituted by HVA, and that constituted by 3,4-dihydroxyphenylacetic acid (DOPAC) is exceedingly sensitive to emotional stress and to factors such as body temperature so that it is possible to see an increase in the levels of one of the metabolites without having an increased turnover of DA.

#### NA turnover in the heart

The significantly decreased turnover of both endogenous NA (formed from  $^{14}\text{C}$ -tyrosine) and exogenous  $^3\text{H}$ -NA in the hearts of chronically exercised rats suggests that the flow of impulses in the cardiac sympathetic nerves is decreased in trained rats. This is so because other explanations for the changed turnover rate can almost certainly be excluded. Firstly the decrease in NA turnover is not likely to be accounted for by super-efficient, almost 100%, reuptake of released NA since the uptake and retention of exogenous NA into the sympathetic nerves in the hearts of chronically exercised animals is in fact significantly lower than that seen in untrained rats (paper II, Fig. 2, this Chapter); this fact in itself might indicate that the cardiac sympathetic nervous activity is lower in the trained rats since it has been claimed that increases in impulse flow lead to a higher degree of reuptake (Haggendorf and Malmfors, 1969). Secondly an effect on turnover rate of the type seen in these experiments cannot be explained by a decrease in intraneuronal metabolism alone, since this contributes quantitatively very small proportion of the total transmitter turnover as seen by the insignificant NA turnover in decentralized organs (Sedvall *et al.* 1968, Swedin 1970). Thus it appears that chronically exercised rats display genuine and marked decrease in the activity of cardiac sympathetic nerves.

There have been several suggestions that newly synthesized NA may be preferentially released and that there may be different pools of NA with nerve endings (Trendelenburg, 1961; Koppi *et al.* 1968; Sedvall *et al.* 1968; Thierry *et al.* 1971; Stjerna, 1971). An alteration in the distribution of NA between the hypothetical "functional/ready" and "storage" pools in cardiac sympathetic nerves in trained rats, so that newly synthesized  $^{14}\text{C}$ -NA is used for transmission for a shorter period than controls and thus becomes stored in a "passive" pool earlier, could theoretically lead to an apparent decrease in turnover rate since the period studied begins 2 h after the injection of the labelled precursor. Such a mechanism is extremely unlikely to account for the non-detectable turnover of the hearts of trained rats found in the  $^{14}\text{C}$ -tyrosine study since the turnover study using exogenous  $^3\text{H}$ -NA showed that like some non-detectable turnover of cardiac NA occurred in chronically exercised rats during day time. If exogenous NA is distributed unequally within these hypothetical pools it is predominantly labelling the rapidly exchanging "pool" in the heart as discussed by Lammier and Sælier (1974). Furthermore the same non-detectable turnover of cardiac NA in trained rat is found in the 0-4 h interval when the turnover rate is estimated by synthesis inhibition by  $\alpha$ -methyl-*p*-tyrosine method which is not of course influenced by any unequal distribution within various pools (see Chapter 5). Lastly these findings are also corroborated by the decreased urinary excretion of NA in the trained rats (see Section D).

It may seem improbable that at rest there should be no significant sympathetic nervous activity at all in the hearts of trained rats but it should be remembered that nocturnal species such as the rat spends most of the day sleeping and thus in a true state of maximal rest. Lammier and Sælier (1973, 1974) have shown that during the day the turnover of NA is significantly lower than during the night, the period of most activity. Indeed if the data from the  $^3\text{H}$ -NA experiment (paper II) is divided in a "day" part, consisting of the 4-12 h interval and a "night" part consisting of the 12-18 h interval, one can discern the same tendency in that study. While the  $t_{1/2}$  of cardiac NA in control rats is 18.1 h during "day" it is 16.1 h during "night" (i.e. day value), whereas in the trained rats the  $t_{1/2}$  during "day" is 19.7 h (82 days different from control  $p < 0.001$ ) compared with  $t_{1/2}$  of 31.2 h during

night (n.s. v. day value) (see Fig. 2).

**Relative bradycardia** trained rats compared with control rats parallels with exercise-induced elevation of heart rate (Ekblom *et al.* 1973) and similarly the turnover rate of cardiac NA remains lower than that of control rats both during continuous and intermittent exercise (see Fig. 2). Thus the half-life of cardiac NA during continuous swimming is 2.6 h in trained rats as compared with half-life of only 1.4 h in the controls ( $p < 0.01$ ) and during intermittent swimming the half-lives were 6.1 h and 3.7 h for chronically exercised rats and controls, respectively ( $p < 0.05$ ).

It seems likely that at least part of the reason for the decrease in sympathetic tone in trained animals is the cardiac hypertrophy associated with larger stroke volume (references see Chapter 1 and Section II of this Chapter) which would lead to a lower heart rate being necessary to maintain any given cardiac output. However it is possible, considering the changes in NA content and total transmitter turnover that occurs in the brain of trained rats, that the brain is actively involved in maintaining this low sympathetic tone in the heart, spleen and probably in the vasculature (see Sections D and F) as opposed to maintaining it only as the basis of unchanged cardiovascular reflexes. The finding that trained rats have normal blood pressure (Craws and Aldinger 1967, Ljungqvist and Unger, 1973) suggests that the total peripheral resistance is more or less unaltered and might thus indicate that the role of the brain is passive one. Whether or not the brain plays an active or passive role in instigating decreased sympathetic tone in chronically exercised animals the maintenance of this decreased sympathetic nervous activity is however probably inversely related to, and dependent upon, the nervous activity of some noradrenergic pathway(s) in the brain as discussed in Chapters 5 and 7.

#### *Noradrenaline turnover in the spleen*

Vascular adjustment during heavy exercise involves an increased sympathetic nervous activity in the spleen (Gordon *et al.* 1966<sup>b</sup>, Sheldon *et al.* 1973) leading to vasoconstriction of the spleen which may function as reservoir of blood in some species. That this is true in rats as well is shown by 13% decrease in spleen weight that occurs after 3 h period of swimming (unpublished observation). In view of this, and of the increased sensitivity to the vasoconstrictor action of NA found in chronically exercised rats (see Section G) it is hardly surprising that the spleen in trained rats shows an adaptive decrease in sympathetic tone just as the heart does. Furthermore this decrease in sympathetic tone in chronically exercised rats probably occurs generally in the vasculature as well (see discussion in Section D).

#### CONCLUSION

In summary therefore chronically exercised rats exhibit reciprocal changes between activity in central and many peripheral noradrenergic neurons: there is an increase in total transmitter turnover in the brain, presumably reflecting increased nervous activity and decrease in NA turnover in many peripheral organs, notably the heart, the spleen and probably the vasculature.

### SECTION II EFFECT OF CHRONIC EXERCISE ON EFFECTOR ORGAN RESPONSE TO CATECHOLAMINES: A STUDY ON PERFUSED HINDBODY AND ISOLATED HEART

*(Paper IV and unpublished data)*

Chronically exercised rats exhibit decreased urinary excretion of NA (papers II, III, Section D) and decreased turnover rate of NA in organs such as heart and spleen (papers II, III, Section F). The

possibility that these adaptive changes in activity of the sympathetic nervous system are reflex-mediated following an increased sensitivity of effector tissues to the actions of NA has been studied in two important effector organs namely the vascular bed and the heart. The vascular bed was studied in the perfused hindbody preparation as described by Folkow *et al.* (1970) with minor modifications, and the heart in the isolated heart preparation of Langendorff (see Chapter 2 for details).

## RESULTS (see Table 3 and Figs. 3 and 4)

### *Vascular bed*

Dose-response curves for the vasoconstrictor action of NA were obtained by plotting the steady-state pressure level for each stepwise rise in NA-concentration in the perfusion fluid as pressure versus  $\log_{10}$  dose NA. A dose-response curve was fitted by eye to the experimental points and the  $M_{50}$  (see below) and the "120-dose" (see below) for each individual curve was determined as the bases for statistical comparison between the two groups. In Fig. 3 the individual points are the means ( $\pm$  S.E.M.) of all individual pressure responses recorded at the various dose levels for each group.

In a first series of experiments the NA solution used for the infusion had ascorbic acid added to protect the NA from oxidation during the course of the experiment; this was however not sufficient and in the series on which the dose-response curve seen in Fig. 3 is based 2  $\mu$ M diethylenetriamine-ethylenedisuccinate (EDTA) was present as well and the gave satisfactory protection against oxidation of the NA. As far as the data on perfusion pressure at maximal distention, maximal pressure response and  $M_{50}$ -pressure (see below for definition) are concerned the two series gave almost identical results and thus in these respects the results from the two series were pooled to give the data in the first part of Table 3.

$M_{50}$ -dose is the dose at which 50% of the maximal pressure response is obtained. This is not the same as the  $ED_{50}$ -dose as discussed by Folkow *et al.* (1970) since it corresponds to 50% of the maximal increase in flow resistance and not to 50% of the maximal contraction of the individual smooth muscle cell, as the increasing wall/tumen ratio of the constricting vessels more and more potentiates the reduction in size of the lumen resulting from a given degree of smooth muscle shortening. Thus the true  $ED_{50}$ -dose is lower than the  $M_{50}$ -dose (see Folkow *et al.* 1970). Since the maximal pressure response in the vascular bed of trained rats is higher than that seen in control rats, further entry the "120-dose" has been introduced to enable direct comparison of the physiologically relevant sensitivity of vascular smooth muscle. The "120-dose" is defined as the concentration of NA in the perfusion fluid that maintains perfusion pressure of 120 mm Hg. This pressure level was arbitrarily chosen as it is roughly on the middle of the steeply-rising, linear part of the dose-response curves as well as being within the physiological range.

As can be seen from Table 3 and Fig. 3 there was no change in the perfusion pressure at maximal distention or in the slope of the linear part of the dose-response curve, in perfused hindbodies of chronically exercised rats compared with those of control rats. On the other hand there was a highly significant 14% increase of the maximal pressure response with consequent increase in maximal perfusion pressure obtained in the vascular bed of chronically exercised rats. Furthermore the dose-response curve to NA in perfused hindbodies of chronically exercised rats shows parallel shift to the left when compared with controls, resulting in a highly significantly lower  $M_{50}$ -dose and mean 120-dose in the trained group. As result of this parallel shift, the vascular bed of chronically exercised rats needs 38% lower NA concentration than that required by the vascular bed of controls in order to maintain a given perfusion pressure in the range of 60–160 mm Hg; thus the sensitivity to the vasoconstrictor actions of NA is markedly increased in trained rats.

**Table 3.** The effect of regular exercise on the pressure response and the sensitivity to the vasoconstrictor action of noradrenaline in the perfused hindbody of the rat and on chronotropic and inotropic responses to noradrenaline in the isolated rat heart.

Values given are means  $\pm$  S.E. with the exception of the slopes of the linear part of the dose-response curve which are given as means  $\pm$  S.D.

Symbols: C = control, T = trained, k = slope (log<sub>10</sub> base) of the dose response curve.

	Perfused hindbody		p-value C v. T
	Controls (n = 14)	Trained (n = 14)	
Pressure at maximal dilatation (mm Hg)	26 $\pm$ 1	25 $\pm$ 2	n.s.
Maximal pressure (mm Hg)	202 $\pm$ 3	225 $\pm$ 3	p < 0.001
Maximal pressure response (mm Hg)	176 $\pm$ 3	200 $\pm$ 3	p < 0.001
M <sub>50</sub> -pressure (mm Hg)	114 $\pm$ 2	124 $\pm$ 5	n.s.
	Controls		p-value C v. T
	(n = 7)	Trained (n = 11)	
Slope (k-value)	189 $\pm$ 16	186 $\pm$ 14	n.s.
M <sub>50</sub> -dose ( $\mu$ M NA/ml)	0.96 $\pm$ 0.04	0.68 $\pm$ 0.05	p < 0.001
120-dose ( $\mu$ M NA/ml)	1.07 $\pm$ 0.06	0.66 $\pm$ 0.04	p < 0.001
	Langendorff heart		p-value C v. T
	Controls	Trained	
Spontaneous heart rate (beats/min)	277 $\pm$ 14 (n = 9)	281 $\pm$ 9 (n = 8)	n.s.
Minimum heart rate (beats/min)	403 $\pm$ 19 (n = 7)	395 $\pm$ 18 (n = 6)	n.s.
Minimum heart rate increase (beats/min)	130 $\pm$ 16 (n = 7)	119 $\pm$ 16 (n = 6)	n.s.
ED <sub>50</sub> dose: chronotropic response ( $\mu$ M NA/ml for 10 sec)	3.10 $\pm$ 0.12 (n = 5)	3.94 $\pm$ 0.55 (n = 4)	n.s.
slope (k-value): chronotropic	42 $\pm$ 18	50 $\pm$ 18	n.s.
M <sub>50</sub> dose: inotropic response ( $\mu$ M NA/ml for 10 sec)	1.96 $\pm$ 0.33 (n = 4)	1.97 $\pm$ 0.43 (n = 3)	n.s.



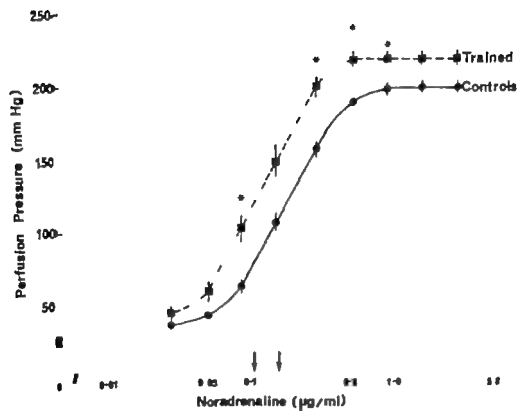


Fig. 3 Dose-response curve to noradrenaline in the perfused hindbody of trained and control rats. Symbols see figure.

The values given are means and the vertical bars represent the S.E. (control 7 trained rats 8).  
denotes different from control  $p < 0.05$     different from controls  $p < 0.01$     different from controls,  $p < 0.001$     ♦♦ denotes different from control  $p < 0.02$ . It is that when larger number of experiments are pooled, the difference in maximal pressure obtained in the two groups is highly significantly different (see Table 3). The two arrows indicate the  $M_{50}$ -dose which is also highly significantly different in the two groups (see Table 3).

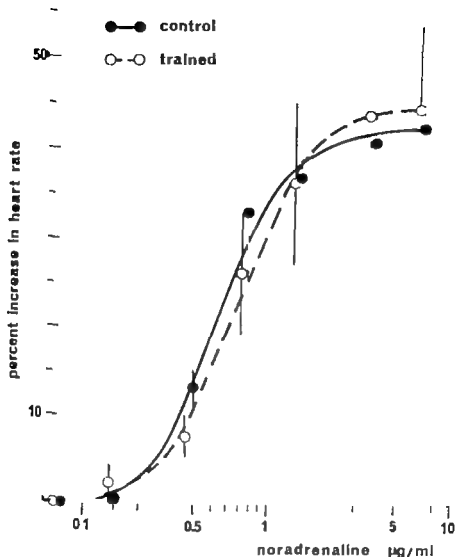


Fig. 4 Chronotropic response to noradrenaline in the isolated rat heart perfused by Langendorff procedure

The values given are means  $\pm$  S.E. (Controls = 5; trained = 4). Curves are fitted by eye to the experimental points and the calculated mean ED<sub>50</sub> dose. Note that the doses employed in the two experimental groups are not identical (due to the 7% difference in perfusion rate) which largely accounts for the difference between the experimental points of the two groups on the steep rising part of the dose-response curve. Furthermore, note that noradrenaline was given in the concentrations indicated as 10 second static infusion only.

## Heart

The intrinsic heart rate of isolated hearts perfused by the method of Langendorff showed a considerable individual variation in both groups but the means were not different (Table 3). The maximal heart rate also varied considerably between individual hearts, but there was no systematic difference between hearts of chronically exercised rats and those of control rats (Table 3).

There was no significant difference in the  $ED_{50}$ -dose for the chronotropic response to NA between the two groups (Table 3). The fact that the mean value in the trained group is a bit higher is because a single observation had a considerably higher value (5.80  $\mu M/ml$ ) than the rest. Preliminary observations (unpublished) have also suggested that there is no difference between hearts from control rats and trained rats in the chronotropic response to isoprenaline. In order to minimize the influence of the large individual variations in the intrinsic heart rate leading to a wide scatter of heart rates obtained in response to a given dose, the composite dose-response curve of the chronotropic response to NA (Fig. 4) is plotted as the % increase of intrinsic heart rate versus log dose of NA rather than as absolute heart rates. This did not, of course, alter the relations between the results in the two groups, i.e. in the actual configuration or slope of the dose-response curves. As can be seen from Fig. 4 the slopes of the dose-response curves, as fitted by eye to the experimental observations, are about the same for the hearts of both groups of rats. This is also confirmed by a least square analysis of the observations on the linear part of the two dose-response curves.  $K_i$ -values  $\pm S.D.$  controls  $42 \pm 18$  trained  $50 \pm 16$ . Furthermore, there is no significant lateral shift of the dose-response curve of the trained group.

The main object was to study the chronotropic responses of the isolated heart. It was therefore decided to record the contractions of the heart via a balloon inserted into the left ventricle and inflated *in situ*, the pressure in the balloon being monitored by a pressure transducer as the isolated rat heart is extremely sensitive to trauma and procedures such as connecting strain-gauges to the cardiac muscle (which gives a better record of inotropic responses) leads to such a high incidence of ventricular arrhythmias that it is impossible to study chronotropic responses. Intracardiac balloon pressure is obviously an indirect and less than ideal way of recording the contractile force. However, in the experiments where the balloon position remained stable throughout the experiment, clear dose-response curves could be obtained by plotting the % increase in peak intra-balloon pressure versus  $\log_{10}$  dose of NA. This sort of curve cannot, of course, be used to obtain a true  $ED_{50}$ -dose but should still be valid for comparisons of relative sensitivity. Using the data in the experiments with stable balloon-position the mean  $M_{50}$ -dose (50% of maximal response) for the inotropic response to NA was found to be practically identical in the two groups (Table 3). Furthermore, preliminary observations (unpublished) have suggested that there is no difference between hearts from trained rats and from controls in the sensitivity to the inotropic action of isoprenaline.

In summary, there appears to be no significant change in sensitivity to the chronotropic and inotropic actions of NA in the hearts of chronically exercised rats.

## DISCUSSION

## Isolated heart

The heart like any other sympathetically innervated tissue develops prejunctional denervation supersensitivity due to removal of neuronal uptake of NA, but it is controversial whether any postjunctional supersensitivity (of the decentralisation-type) occurs. Dempsey and Cooper (1968) claim that surgically denervated hearts develop prejunctional denervation supersensitivity only and Haerster *et al.* (1968) suggest that postjunctional supersensitivity is also absent after chemical sympathectomy of the heart by 6-hydroxydopamine treatment. On the other hand, Westfall and Fleming (1968) claim

that pretreatment with reserpine leads to postjunctional type I supersensitivity which is characterised by partial depolarisation of atrial cardiac muscle cells (Flaming *et al.* 1975) and which they suggest is equivalent to decentralisation supersensitivity. These authors were however only able to demonstrate these changes with some bath media such as Locke's solution but not with a modified Krebs-Henseleit solution. Furthermore it cannot be excluded that the effects observed by Flaming and co-authors are due to non-specific direct pharmacological effects of the reserpine treatment on the cardiac muscle cell rather than a decentralisation type of postjunctional supersensitivity. In summary therefore, there are considerable doubts as to whether the heart is normally capable of developing a postjunctional type of supersensitivity and thus it is not surprising that chronic physical exercise failed to alter the sensitivity of isolated hearts to NA.

The claim of Crews and Aldinger (1967) that there was decreased sensitivity to the inotropic actions of adrenaline in hearts of trained rats can probably be disregarded as it was based not on a proper dose-response curve but on the response to a single I. dose, and was not supported by any figures or statistics. Likewise the claim of Pavluk and Friedl (1975) that trained human subjects showed smaller blood pressure response to NA, but an unchanged blood pressure and heart rate response to adrenaline is impossible to evaluate as again the claim is based only on response to single I. dose, without proper dose-response curve. This is not a valid approach since the actual concentration of the drug reaching the organ studied is dependent among other factors on body weight (not given) the percentage of body weight made up by water (not known likely to be different in trained and untrained subjects, see Section B) and in particular the blood volume (known to be increased in trained subjects, see Sjostrand, 1955). Furthermore the response studied is summation of the effects of unknown concentrations of the injected catecholamine with circulating levels of catecholamines (likely to be different in trained and untrained subjects, see Section D) and with the activity of the autonomic nerves (also likely to be different, see Section F).

It is probably fair to summarise, therefore, that any valid conclusion about possible alterations of receptor sensitivity in trained animals must be based on studies on isolated organs.

Sutton *et al.* (1967) Lin and Horvath (1972) and Ekblom *et al.* (1973) all interpreted the lower heart rate found in chronically exercised humans and rats after simultaneous treatment with atropine and propranolol as being due to lower intrinsic heart rate in the trained individuals. This finding, however, is more likely to be due to incomplete  $\beta$ -blockade as the atropine-induced increase in heart rate was not abolished either in the study of Sutton *et al.* (1967) or in that of Ekblom *et al.* (1973). In the case of Lin and Horvath's study (1972) the dose of propranolol given to the rats (8 mg/kg BW i.p.) is certainly not enough to block very substantial increases in heart rate caused by the increased cardiac sympathetic nervous activity induced simply by handling the rat (unpublished observation). Furthermore in my experiments the intrinsic heart rate of isolated Langendorff hearts (see Table 3) as well as the heart rate in rats given atropine plus the ganglion blocker chlorisondamine (unpublished observation) is the same in control and chronically exercised rats. Using isolated atria from rat hearts Bolter *et al.* (1973) reported slower intrinsic rate in the atria from trained rats. There are at least two factors that could explain the discrepancy with my findings, firstly they studied their atria in an organ bath at non-physiological temperature only 30°C, secondly the isolated atrium is undoubtedly more traumatised preparation than Langendorff heart, particularly when as in the study of Bolter and co-workers, the atria are mounted under tension, and it is possible that the slower rate of the atria of the trained rats is due to release of acetylcholine from the atrial tissue since trained rats have considerably increased acetylcholine content in the atria (Herrlich *et al.* 1960).

Some authors have suggested that the maximal heart rate might be somewhat lower in trained humans (Ekblom *et al.* 1973; Bronson *et al.* 1976) however these authors simply studied the heart rate at the maximally tolerated work load, and no figures were given to prove that the heart rate had actually reached a plateau where no further heart rate increase was possible. In my own study there

was no suggestion of a significant difference between the maximal heart rate in isolated hearts from trained and control rats.

### *Vascular bed*

The perfused hindbody preparation as described by Folkow *et al.* (1970) contains a vascular bed that consists largely of the normal resistance vessels in the body. Therefore, alterations in the sensitivity to NA of this preparation represent changes with a direct physiological relevance for regulation of the blood pressure. The degree of increased sensitivity found in trained rats is such that a 39% lower concentration of NA than in control rats is required to produce the same increase in perfusion pressure; this is remarkably paralleled by the finding that trained rats at rest excrete 33% less NA than control rats (see Section D). Thus, the reduced release of NA from vascular nerves in trained rats should be just adequate to maintain normal blood pressure as has indeed been found experimentally (Crawe and Aldinger, 1967; Ljunqvist and Unger, 1972).

Altered dose-response curves to NA in vascular preparations such as the perfused hindbody can be caused by a variety of changes, for example: 1) Hypertrophy of the smooth muscle layer of the tunica media of the vessel wall; 2) Increased contractility per unit of contractile tissue; 3) Changes in the size of the vascular bed; 4) Altered uptake of NA into sympathetic nerves; 5) Altered extraneuronal uptake of NA; 6) True change in the sensitivity of smooth muscle to vasoconstrictor properties of NA mediated either by an alteration in the properties of the  $\alpha$ -receptors, the  $\beta$ -receptors or both. These various possibilities will now be discussed in turn to see which is the most likely to account for the changes found in chronically exercised rats.

It is known that hypertrophy of the media in the arterial vessel wall leads to a more steeply rising dose-response curve as seen for instance in spontaneously hypertensive rats (Folkow *et al.*, 1970). This occurs irrespective of whether or not the hypertrophy of the media encroaches on the lumen at maximal dilatation because the same degree of shortening of the individual smooth muscle cell will lead to a more pronounced decrease in lumen size when the wall/lumen ratio is higher than normal (see Folkow *et al.*, 1968). However, when the hypertrophied muscle does encroach on the lumen at maximal dilatation the perfusion pressure at maximal dilatation is increased, as occurs in spontaneously hypertensive rats (Folkow *et al.*, 1970). We can conclude that hypertrophy of the media has not occurred in the trained rats for two reasons. Firstly, at a identical perfusion rate the perfusion pressure at maximal dilatation was the same as in controls, and consequently there can be no hypertrophy of the vessel wall that encroaches upon the lumen at maximal dilatation. Secondly, the slopes of the dose-response curves were the same in both groups.

An increased ability to develop active tension per unit of contractile tissue in blood vessel of unaltered design would, according to Folkow *et al.* (1970), increase both the slope of the curve and the maximal pressure response leading to an unchanged  $M_{50}$ -dose. Thus such a hypothetical mechanism cannot explain the parallel shift in the dose-response curve and the lowered  $M_{50}$ -dose found in the trained rats.

A decrease in size of the vascular bed would lead to an increased perfusion pressure at maximal dilatation and a steeper slope as the flow per resistance vessel would be higher and neither of these changes are observed here.

Blocking neuronal uptake of NA (uptake<sub>1</sub>) not only shifts the dose-response curves to 1-NA to the left but also decreases the slope of the curve (see review by Trendelenburg, 1972). Therefore diminished neuronal uptake of NA seems unlikely to be the cause of the observed shift in dose-response curves in chronically exercised rats, since the slopes of the dose-response curves in the two experimental groups are virtually identical.

The presence of steroids in organ bath or perfusing medium has been shown to potentiate the

action of catecholamines by impairing extraneuronal uptake (uptake<sub>2</sub>) of NA (Kahnar 1989; Hansen and Salt 1970). In this experiment however the perfusion medium contained no starvoxin and, furthermore, it might be expected that an impaired uptake<sub>2</sub> would result in a less steep dose-response curve just as seen with impaired uptake<sub>1</sub> (see above).

It therefore appears that there is only one satisfactory explanation for the parallel shift in dose-response curve found in trained rats, namely that there has been a true increase in vascular smooth muscle sensitivity to the vasoconstrictor action of NA. As NA is not only a potent  $\alpha$ -agonist but also a  $\beta_2$ -agonist, albeit rather weak one (Furchgott, 1972) increased vasoconstrictor response could be due either to a decreased sensitivity to  $\beta_2$ -actions or an increased sensitivity to  $\alpha$ -agonist actions.

Some evidence that decreased sensitivity of vascular  $\beta_2$ -receptors can occur in some experimental conditions is provided by the work of La Blane *et al.* (1972) who have shown that in rats treated chronically with isoprenaline the acute fall in blood pressure following a dose of isoprenaline is much reduced or even abolished. It is unfortunate that these authors did not publish a proper dose-response curve. Their experimental conditions are similar to the present study in so far as daily exercise provides a daily exposure of the blood vessels to high concentrations of NA, which could conceivably have the same effect on the  $\beta_2$ -receptor of vascular smooth muscle as daily administration of isoprenaline. Furthermore, Spier and Weinstock (1974) suggest that isolation of animals, which leads to decrease in sympathico-adrenal activity (Welch and Welch, 1968, 1969) gives rise to an increased sensitivity of vascular  $\beta_2$ -receptors to both isoprenaline and NA, resulting in decreased pressor response to NA. In the experiments of Spier and Weinstock (1974) low doses of NA not infrequently actually led to a fall in blood pressure in isolated, but not in group-housed, rats. This observation suggests that in the isolated rat the threshold for the  $\beta_2$ -agonist actions of NA is lower than the threshold for  $\alpha$ -agonist action. Normally this difference in threshold sensitivity is only revealed when adrenaline is used (see review by Neil 1975). A change in sensitivity to  $\beta_2$ -mediated responses with an unaltered  $\alpha$ -receptor sensitivity would then be expected to lead to an altered slope of the dose-response curve in isolated rats with a steeper rising curve. The data of Spier and Weinstock seem to confirm that such a change in slope occurs. Since the slope of the dose-response curve was unaltered in my trained rats it is not likely that decreased sensitivity to the  $\beta_2$ -action of NA is the mechanism of the observed changes in sensitivity.

We are left, by elimination of the other explanations, with the possibility that quantitative and/or qualitative changes in the  $\alpha$ -receptor population or in its link with the contractile response are the most likely mechanisms of the observed increased sensitivity to the vasoconstrictor actions of NA seen in trained rats. What then could be the trigger for such a change in the  $\alpha$ -receptor mediated response in vascular smooth muscle? It is known that decentralisation of sympathetic neurons leads to a post-receptor type of supersensitivity to the actions of NA (see review by Trendelenburg, 1972). As the sympathetic nervous activity at rest is lower than normal in at least the heart and the spleen (Section F) and probably also in the vasculature (see Section D) of trained rats, one might speculate that this lowered sympathetic tone might lead to a decentralisation-type of hypersensitivity. However supersensitivity following surgical decentralisation takes more than 48 hours to become measurable and does not reach maximum until four weeks or more after decentralisation (see Trendelenburg, 1972). Since the trained rats were submitted to daily bouts of exercise throughout the experimental period, there were daily increases in their sympathico-adrenal activity sufficient to lead to excretion over 22 hours of an amount of NA in the urine that was of the same order as (paper III), or larger than (paper II) the amount excreted by the resting control rats. Therefore it would appear that sympathetic nervous activity in chronically exercised rats is not depressed to a sufficient degree for long enough periods for decentralisation supersensitivity to occur.

That the opposite phenomenon can occur namely that increases in receptor sensitivity can occur in response to increased exposure of the receptor to its agonist appears to have been shown for the

metabolic "cologenic" action of NA both in cold-acclimated rats (Hale and Carlson, 1957 for further references see Chapter 4) as well as in rats treated with repeated NA injections (Le Blanc and Pouliot, 1964) and for the isoprenaline-induced increase in  $O_2$ -consumption (Le Blanc *et al.* 1972). Such a mechanism, namely potentiation of the  $\alpha$ -receptor response of vascular smooth muscle occurring as a result of increased exposure to physiological  $\alpha$ -agonists (NA and adrenaline) would provide a convenient hypothesis to explain the findings: the trained group as these rats are subjected to bouts of markedly increased sympathetic nervous activity as well as increased secretory activity of the adrenal medulla, during their daily exercise periods.

There is however one further hypothetical mechanism that deserves consideration. Physical exercise leads to increased secretion of corticosteroids from the adrenal cortex (for ref. see Chapter I and Section B) and it is possible that such changes in the endocrine status of the animal can alter the structure and/or the number or function of for example vascular smooth muscle  $\alpha$ -receptors. Changes in effector organ sensitivity to NA following prolonged changes in circulating steroid levels (changes that are present in isolated organs perfused by steroid free medium) have been reported to occur in both directions. Firstly pregnancy (Tessier and De Jong, 1973; Dogterom and De Jong, 1974) and chronic progesterone treatment (Dogterom and De Jong, 1974) has been showed to lead to reduced vascular sensitivity to the vasoconstrictor actions of NA. Secondly elevated levels of plasma corticosterone increase the maximum contractile response to NA, and increase both sensitivity and maximum contractile response to acetylcholine in the anococcygeus muscle of rat (Gibson and Pollock, 1976).

The latter authors studied the increase in sensitivity and maximal contractile response of the anococcygeus muscle of the rat occurring after short-term treatment with reserpine (after morphine withdrawal and after thyroidectomy). They found the responses in each case to be qualitatively similar and to be prevented by adrenalectomy and concluded that the mechanism behind the increase in sensitivity in all these experimental conditions was the increase in plasma corticosterone levels known to occur after these various treatments (see Gibson and Pollock, 1976). Carrier and co-workers have shown that short-term (24-48 hours) treatment with reserpine leads to an increase both in sensitivity and in maximal contractile response to NA in aortic and femoral artery strips of dogs and to increased pressor responses to NA in rats (see Carrier, 1976). They attributed these changes to decentralization supersensitivity but as they occur more rapidly than true decentralization supersensitivity (see Trendelenburg, 1972) the increases in sensitivity and in maximum response may well be associated with the rise in plasma corticosterone levels that persists for a prolonged period following reserpine treatment (Gibson and Pollock, 1976). It is noteworthy that the qualitative changes in vascular response to NA provoked by short-term reserpine treatment are exactly the same as those seen in chronically exercised rats and that, as discussed in Section B, physical exertion leads to rise in plasma corticosterone levels.

The mechanism behind the increase in maximum contractile response observed in the vascular bed of chronically exercised rats is not clear. Carrier and co-workers have shown that aortic strips of reserpine pretreated rabbits handle  $Ca^{2+}$  differently from control strips (see Carrier, 1976) and Westfall *et al.* (1975) have shown that tissue concentrations of ATP rise in conjunction with the development of decentralization supersensitivity in the vas deferens, both after denervation, decentralization, and after reserpine treatment. Similar mechanisms could be responsible for the observed increase in maximal contractile strength of vascular smooth muscle in this study.

**Conclusions.** Thus it appears likely that the increase in vascular sensitivity to the vasoconstrictor actions of NA seen in chronically exercised rats is a compensatory response most likely brought about by events occurring during the acute physiological rise of heavy muscular exertion either by the effects of locally high concentrations of transmitter on the receptor site and/or by the accompanying increase in circulating levels of corticosteroid hormones, in the rat particularly corticosterone.

It is frequently assumed that increased sensitivity of an organ always occurs after chronic low

level of stimulus and that a decreased sensitivity may occur after chronically increased stimulus levels (see Fleming, 1975). The vascular bed of the chronically exercised rat appears to form an exception to this rule. In that a regular experimental stimulus which causes the vascular smooth muscle to be exposed to increased release of NA from sympathetic vascular nerves as well as to higher concentrations of circulating NA is nevertheless associated with an *increased* sensitivity of the effector cell. In this experimental condition such a change can be regarded as a useful adaptation since it means that transmitter stores can be better conserved in situations where there is an extreme demand for a sustained increase in vascular tone because the requirement for NA will be decreased in proportion to the increase in postsynaptic sensitivity.

## CONCLUSIONS to Chapter 3

(1) Daily exercise leads to a reduction in weight gain which is not seen in intermittent (3 times/week) exercise.

(2) Both daily and intermittent exercise leads to the same degree of cardiac hypertrophy provided the exercise load per session is the same. If the training is started early (i.e. before the cardiac cells have lost their capacity for mitoses) the heart of the exercised rat remains larger than that of sedentary control even 6 months after cessation of training.

(3) Intermittent exercise causes the same increase in adrenal gland weight as daily exercise does.

(4) Trained rats exhibit a decrease in weight of the spleen; this is probably due to a reduction in lymphoid tissue secondary to the exercise-induced elevation of plasma corticosterone level.

(5) The stores of transmitter in noradrenergic neurons in the brain, heart and spleen are increased by daily swim training and cardiac sympathetic neurons in trained rats probably have an increased maximal capacity for NA synthesis as well. It is suggested that this is part of a generalized adaptive response of the neuron to prolonged increases in impulse flow, a response which includes increased activity of the enzymes involved in NA synthesis and probably an increase in number of storage vesicles as well. In the heart it is furthermore possible that an actual neoformation of noradrenergic nerve terminals, occurring together with the formation of new capillaries, may contribute to the increase in transmitter content. Whereas some increases in cardiac NA content persist even 6 months after cessation of training, the elevation of NA content in the spleen appears to be transient, disappearing towards the end of a 17 week training programme.

The increase in cardiac NA content and concentration is probably less pronounced in rats trained by intermittent (3 times/week) exercise than that seen after daily exercise.

(6) The adrenal medulla also shows an adaptive increase in the catecholamine content of the chromaffin cells following daily exercise and this is more pronounced in the NA containing cells. In intermittently exercised rats the increase in adrenaline content is similar to that seen after daily exercise, whereas the increase in NA content is considerably smaller than that seen in rats trained daily.

(7) The amount of NA secreted in the urine is lower in the rat trained by daily exercise than in sedentary rat, both at rest and during exercise and it is suggested that this reflects a decreased activity in sympathetic nerve terminals in the vasculature. The amount of adrenaline secreted in the urine on the other hand, is the same in trained and sedentary rats during resting conditions. After exercise, however, the increase in urinary adrenaline excretion is much smaller in trained rats. Intermittently exercised rats have urinary excretion of both NA and adrenaline that, after an exercise session, is intermediate between the values seen in trained and sedentary rats. It is argued that this suggests that the reduction in the exercise-induced secretion of adrenaline from the adrenal medulla of trained rats is not simply due to an "adaptation to the emotional" stress of swimming but reflects further adaptation perhaps in the metabolic responses to adrenaline.



(8) Both central and some peripheral noradrenergic neurons of trained rats show adaptive changes in nervous activity but in opposite directions. Thus the amount of NA turning over in the brain is increased in trained rats at rest, whereas the turnover of NA is decreased in the sympathetic neurons of the heart and the spleen. During conditions of minimal physical activity there is in fact no detectable turnover of NA in the heart or spleen and it must be assumed that this represents complete inhibition of sympathetic tone.

During both continuous and intermittent swimming the turnover of NA in the heart is markedly increased compared with the resting values but remains lower in trained rats than in untrained rats. The dopaminergic neurons of the brain do not show an increased DA turnover during exercise, in contrast with the increased turnover seen in noradrenergic neurons in the brain, and perhaps because of this there is no alteration of DA turnover in the brain of trained rats at rest.

(9) The isolated heart from a trained rat shows no alteration in intrinsic heart rate, maximal heart rate or sensitivity to the chronotropic and inotropic actions of NA. On the other hand, the vascular bed of trained rats shows adaptive changes consisting of an increased maximal constrictor response and an increased sensitivity to the vasoconstrictor actions of NA, without any indication of hypertrophy of the smooth muscle layer in the vascular wall. It is argued that this represents an increase in sensitivity to the  $\alpha$ -receptor mediated response brought about by the effects on the vascular smooth muscle either of repeated bursts of exercise-induced NA release or of the exercise-induced elevation of plasma corticosterone levels.

# A COMPARATIVE STUDY OF COLD-ACCLIMATION AND CHRONIC PHYSICAL EXERCISE (paper III)

## HISTORICAL BACKGROUND

Since homeothermic organisms must maintain their body temperature at an optimal level the exposure to a cold environment means that such an animal must decrease its heat losses by increasing insulation or increase its heat production, or both. Short-term decrease in heat loss can be achieved only by pilo-erection and by superficial vasoconstriction. As already recognised by Claude Bernard (1871) quoted by Leduc (1961) there are two main sources for the increased production of heat in response to cold exposure firstly increased muscular activity and secondly other chemical processes unrelated to muscle work. When an animal is first exposed to a cold environment (in the temperature range of 1 to 5 C) there is a marked increase in muscle tone and regular shivering commences in order to augment heat production as the exposure becomes chronic, heat production remains elevated but the shivering declines and after about 4 weeks it disappears altogether (Seilers *et al.* 1954 Hart *et al.* 1966). Such an animal has acclimated to the cold environment by increasing its capacity for non-shivering thermogenesis and subsequently non-shivering thermogenesis takes precedence over shivering thermogenesis as the preferred method of increased heat production.

### *Role of the sympatho-adrenal system in cold-acclimation*

Early work of Hartmann *et al.* (1923) and Cannon *et al.* (1927) showed that the circulating levels of catecholamines were increased by cold exposure, and these workers suggested that the adrenal medulla was a major source of these catecholamines; however the studies of Cannon and co-workers on surgically sympathectomized rats (see Cannon 1932) also demonstrated that their ability to maintain their body temperature on cold exposure was impaired and thus suggested a role for the sympathetic nervous system as well. Subsequently it has been confirmed that the secretory activity of both the sympathetic nervous system and the adrenal medulla is increased by cold exposure. The sympathetic nervous system largely serves as the "first line of the defence" with perhaps supportive role for the adrenal medullary secretion of adrenaline in the maintenance of normal blood glucose levels. A marked increase in adrenal medullary secretion of adrenaline represents the "second line of defence" when increased activity of the sympathetic nervous system has failed to maintain homeostasis (see reviews by Leduc 1961 Henne-Hagen, 1978). The increased activity of the sympathetic nervous system in cold exposure serves several purposes. First, increased sympathetic nervous activity is responsible for improving the insulation by pilo-erection and by superficial vasoconstriction. Secondly it is involved in the necessary cardiovascular readjustments with increased sympathetic nervous activity in the heart (Olivero and Björne 1965 Gordon *et al.* 1966<sup>b</sup> Bhagat and Friedman, 1969) and redistribution of the increased cardiac output in general from skin to viscera (Lansky and Hart, 1968). Thirdly it is involved in non-shivering thermogenesis both by actually initiating the process and by mobilizing substrate (see reviews by Henne-Hagen, 1978).

### *Changed calorigenic response*

The initial observation that cold acclimated rats had an enhanced calorigenic response to adrenaline was made by Rang (1942) and confirmed by Hsieh and Carlson (1957), and Svensson (1957), and

subsequent work also showed a striking enhancement of the calorogenic response to NA in cold-acclimated rats (Haleth and Carlson, 1957; Depoos, 1960 and many later authors: see review by Himms-Hagen, 1975). Although the maximal calorogenic response to both NA and adrenaline is increased to an equal degree in cold-acclimated rats, it has been shown that in this species NA is normally the main chemical mediator of non-shivering thermogenesis (see reviews by Ladue, 1961 and Himms-Hagen, 1975). During continued cold-exposure the capacity for non-shivering thermogenesis increases and reaches a maximum after about 4 weeks so that a cold-acclimated rat can maintain its body temperature in a cold environment even when paralyzed by curare, whereas a warm-acclimated animal under the same conditions rapidly becomes hypothermic (Cottle and Carlson, 1956).

Brown adipose tissue hypertrophies in cold-acclimated rats and is assumed to be a site of non-shivering thermogenesis, however the major part of non-shivering thermogenesis is likely to occur in skeletal muscle (see review by Himms-Hagen, 1975).

The calorogenic effect of a standard dose of exogenous NA increases rapidly during the first few days of cold exposure, thereafter it increases more slowly and a plateau is reached after about 30–40 days of cold exposure (Jensky *et al.* 1967) — other words at the same time as they cease to shiver. The size of the maximum metabolic response given by a standard dose of NA is however inversely proportional to the temperature of acclimation, so that the lower the temperature the higher the final maximal calorogenic response. The time course of the acclimation process is however identical at different temperatures, at least within the range  $-1^{\circ}\text{C}$  to  $+15^{\circ}\text{C}$  (Jensky *et al.* 1967). Le Blanc and co-workers have shown that similar increases in calorogenic response to catecholamines with an accompanying increase in cold-tolerance, can be achieved by daily injections of NA (Le Blanc and Pouliot, 1964) or isoprenaline (Le Blanc *et al.* 1972) and it appears, therefore, that it is the increased activity of the sympathetic nervous system that actually triggers the development of the adaptive increase in the total capacity for non-shivering thermogenesis.

#### *Urinary excretion and tissue levels of catecholamines*

The excretion of NA and adrenaline in the urine is increased during acute cold exposure. The elevated urinary excretion of NA persists throughout the duration of cold exposure but as the rats gradually become acclimated, the amount of NA excreted decreases significantly below the level at the onset of cold exposure and finally stabilizes at a value about three times that of controls: the excretion of adrenaline eventually declines to a level about 50–100% above that of warm-acclimated rats at room temperature (see review by Ladue, 1961). As the heat production remains equally elevated this would seem to indicate that an increase in the sensitivity to the calorogenic actions of NA and adrenaline, as well as an increase in the maximal capacity to respond, has occurred in cold acclimated rats. Such an increase in sensitivity has also been demonstrated by Jensky *et al.* (1960).

During acute cold exposure the adrenaline content of the adrenal medulla is usually decreased, within 1 day but after about 6 days of continued cold exposure the adrenaline content is higher than before exposure and in most studies remains elevated throughout cold exposure (see review by Himms-Hagen, 1975). The NA content of the adrenal gland is decreased only by severe cold exposure (Ladue, 1961) and in cold-acclimated rats the adrenal NA content has been variously reported as normal (Ladue, 1961) or elevated (Des Marais and Dugal 1961; Moore *et al.* 1961 and Kurowsky *et al.* 1971). Acute cold exposure also decreases the NA content of heart, liver, spleen, muscle and brown adipose tissue (see review by Himms-Hagen, 1975). However on the 5th day of continued cold exposure, NA concentrations in heart, spleen, liver and muscle tended to be elevated, whereas after complete cold acclimation the organ concentrations of NA were no longer significantly above normal (Ladue, 1961) unfortunately the total organ content of NA was not reported in this study.

Acute cold-exposure increases the rate of NA turnover in the brain (Semonovs, 1969) but the NA

content of brain and hypothalamus is generally reported unchanged after moderate degrees of cold exposure (Moore *et al* 1961; Gordon *et al* 1965<sup>b</sup>; Co and Potkanyak, 1967) and reported as increased (Ingenito and Bonnycastle 1967) after prolonged cold exposure.

### *Organ and body growth in cold-acclimated rats*

Some but far from all previous studies have reported decreased weight gain in cold-acclimated rats (Schwabe *et al* 1938; Haroux and Hart, 1954; Haroux and Gridgeman, 1958; Strjeme and Hammel, 1967) whereas on the other hand cold-acclimated rats display hypertrophy of heart, adrenal glands, thyroid gland, kidney, liver and intestinal tract (Emery *et al* 1940; Haroux and Hart, 1954; Harou and Gridgeman 1958). The decreased body weight is mainly accounted for by reduced skeletal muscle mass, but the weights of total fat tissue, skeleton and spleen are also reduced (Haroux and Gridgeman 1958) and, occasionally total body length has been reported to be slightly below control values (Emery *et al* 1940). It should however be pointed out that reduction in skeletal muscle mass does not occur in wild raptorial mammals which acclimates to cold during the winter and the only significant change in organ weight in these rats is cardiac hypertrophy (Haroux, 1961).

### *Physical fitness and cold-acclimation*

That man may acclimate to cold exposure in the same way as small mammals by increasing his metabolic heat production has been claimed by several investigators (Burton, 1948; Adams and Heberling, 1958; Scholander *et al* 1965). However most studies on cold-acclimation in man have been field studies where increased levels of physical activity have been unavoidable and it was suggested by Adams and Heberling (1958) that many of the findings taken to indicate cold-acclimation in man might have been due to change in physical fitness alone. In support of this suggestion, the same authors reported that after three week programme of physical training the trained subjects maintained higher skin temperatures and had elevated heat production when compared with control subjects in cold test and these observations were partly confirmed by Keatings (1961). Lariv Heberling and Adams (1961) reported that following an extended programme of physical training subsequent period of cold exposure (winter bivouac in Alaska) did not produce any further improvement in cold tolerance and similar conclusions were reached by Lange Andersen (1966).

The inherent difficulty in doing this type of study in humans is the problem of standardizing the cold exposure and achieving strictly comparable control group and these problems are of course much easier solved in laboratory studies on small mammals. One such study dealing with the relationship between physical fitness and cold acclimation demonstrated that chronically exercised rats, like physically fit men, exhibited considerably greater heat production and maintained higher skin temperatures on their feet during cold exposure than control rats did, although during severe cold exposure the chronically exercised rats were not able to maintain their skin temperatures quite as well as cold-acclimated rats (Strjeme and Hammel, 1967).

### *Present experiments*

Thus there are many indications that the process of cold-acclimation has several mechanisms in common with adaptation to chronic exercise and the study (paper III) was designed to compare directly some aspects of the sympathetic nervous system in these two adaptive conditions.

In this study one group of rats was trained by chronic exercise (swimming) and a second group was cold-acclimated at 4°C. These experimental groups were compared with warm-acclimated control rats. In the studies on urinary CA secretion after acute exercise the chronically exercised rats had been sedentary for 48 h and the cold-acclimated rats had been at room temperature for 48 h.

## RESULTS (paper III)

### *Body and organ weights*

There was no change in final body length in any of the groups, but as mentioned earlier the mean body weight of trained rats was 7% less than that of controls, the mean body weight of the cold-acclimated rats was in between these two groups and not significantly different from either group. Both cold-acclimated rats and exercised rats displayed cardiac hypertrophy of the same magnitude, with mean heart weights 7% heavier than that of the control group. Submandibular glands were significantly hypertrophied in the cold-acclimated group compared with both controls and exercised rats, with a weight increase of 13% compared with the control value. The weight of the spleen in cold-acclimated rats was no different from that of controls, whereas the decreased spleen weight in the trained rats was statistically significant only when compared with the cold-acclimated group (-9%). Both trained and cold-acclimated rats displayed hypertrophy of the adrenal gland (+8% and +13% respectively).

### *Catecholamine content of the organs*

The NA content of the brain was unaltered in the cold-acclimated rats (controls  $0.84 \pm 0.02$   $\mu$ g NA,  $n = 12$  cold-acclimated  $0.85 \pm 0.04$   $\mu$ g NA,  $n = 11$  previously unpublished). The total NA content of the heart was significantly increased in both trained (+26%) and cold-acclimated (+18%) rats whereas cardiac NA concentration was significantly increased only in trained rats (+18%). The total NA content in submandibular glands was significantly increased in cold-acclimated rats (+15%), whereas the NA concentration in the gland was not significantly altered in any group. The total content of NA in the spleen was elevated (+26%) in trained rats but decreased (-14%) in cold-acclimated rats. The total content of adrenaline in the adrenal gland was significantly increased only in the trained group (+13%) although the adrenaline content also tended to be somewhat elevated above control values (+10%) in the cold-acclimated group. The adrenal NA content was markedly increased (+32%) in both trained and cold-acclimated rats and consequently the proportion of the total CA content of the adrenal gland that is composed of NA is significantly elevated in both trained and cold-acclimated rats.

### *Urinary excretion of catecholamines*

During resting conditions both trained and cold-acclimated rats excreted significantly less NA in the urine - 23% and - 31% respectively - than did control rats. There was no difference between the urinary excretion of adrenaline between trained rats and controls, but in the cold-acclimated group the excretion of adrenaline was 80% lower than that of controls. After exercise all groups excreted significantly more NA than during rest, but only controls and cold-acclimated rats excreted significantly more adrenaline. However the amounts of NA excreted after exercise were different in the three groups. Thus the trained rats excreted 36% less NA after exercise than the control group and 25% less than the cold-acclimated group, the cold-acclimated rats in their turn also excreted significantly less NA (-15%) than the controls did. When paired observations from the same animal were studied it was found that the net increase in the amount of NA excreted after exercise (i.e. after subtracting the amount of NA excreted during rest period) was of the same magnitude in cold-acclimated rats and controls, but significantly smaller (-42%) in trained animals. During cold stress the excretion of both NA and adrenaline in the urine was markedly increased compared with the resting values in all the experimental groups. The excretion of adrenaline was of the same order of magnitude in all groups, whereas the amount of NA excreted in the urine by both the cold-acclimated and the trained rats was significantly lower than that of the control group - 17% and - 20% respectively.

## DISCUSSION

The findings of trained rats are discussed in detail in Chapter 3 and will only be further discussed here in relation to the observations on the cold-acclimated rats.

### Body and organ weights

In this study the marked inhibition of weight gain in cold-acclimated animals reported by several previous studies (for references see Introduction) is not seen. This is probably mainly due to the fact that in our experiment the severity of the cold stress was somewhat diminished by keeping the cold-acclimated rats in the same way as the controls and exercised rats, i.e. in cages with bedding, 8 to 8 rats per cage thus allowing some protection. Strain differences could also be another influencing factor since crossbred Sprague-Dawley-Wistar rats are much more resistant to inhibition of growth by cold-acclimation than are Wistar rats (Heroux and Hart 1954). Finally it should be recalled that although the body weight of the cold-acclimated rats did not differ significantly from that of the controls it did not differ significantly from the body weight of the trained rats either. This suggests that there is a moderate decrease in body weight of our cold-acclimated rats.

The cold-acclimated rats were found to have the same degree of cardiac hypertrophy as the trained rats, which is hardly surprising in view of the considerable increase in cardiac output during cold exposure found in both warm- and cold-acclimated rats (Lansky and Hart, 1968). Cold exposure to temperature no more severe than  $+10^{\circ}\text{C}$  gives rise to increases in cardiac output, stroke volume and heart rate of the same degree as does running at a speed of 20 m/min in warm-acclimated, untrained rats (Popowa *et al.* 1968) and increased activity in the sympathetic nerves to the heart is shown by the finding that acute exposure to cold as well as to exercise increases cardiac NA turnover (for references see Historical Background).

As mentioned in the Historical Background the influence of cold-acclimation on the size of different organs has been extensively studied. However one organ which seems so far to have escaped attention is the salivary gland. In view of the increased food intake of cold-acclimated rats (Schnebe *et al.* 1938) and the fact that liver and digestive tract have been found to weigh more in cold-acclimated rats (Heroux and Gindoffman, 1958) the finding that the submandibular glands of cold-acclimated rats significantly increase in weight is not very surprising. It is however noteworthy that the total amount of NA in the submandibular glands of cold-acclimated rats is also significantly increased, this maintaining an unchanged transmitter concentration. Catecholamines induce the secretion of amylase and the enlargement of salivary glands occurring in response to tooth amputation is mediated by the sympathetic nervous system, furthermore large doses of isoprenaline cause hypertrophy and hyperplasia of salivary glands in rats (see review by Hamme-Hagen 1972). Thus a reasonable interpretation of these findings would be that the enlargement of the submandibular glands in cold-acclimated rats is related to trophic influence of increased sympathetic nervous activity secondary to increased food intake and/or to cold-acclimation itself.

The increase in weight of the adrenal glands of cold-acclimated rats is in agreement with previous reports (see Historical Background) and this hypertrophy is known to occur within one week of the onset of cold exposure (Heroux and Schonbaum, 1960; Kvetnansky *et al.* 1971) the weight increase of the gland is due to an increase in the number of cells (the zona fasciculata) (Heroux and Schonbaum, 1960).

### Organ catecholamine content

Possible mechanisms underlying the increase in cardiac NA content in chronically exercised have been discussed in detail previously (see Chapter 3 Section C). It is likely that the same

are involved in the increase of the total NA content of the heart found in cold-acclimated rats, since as mentioned above the activity of cardiac sympathetic nerves is increased by cold exposure and since acute intermittent swimming in +15°C water (an experimental stress where cold stress is the predominant feature) causes increased tyrosine hydroxylase activity in rat stellate ganglia and in the heart (Thoenen *et al.* 1973). Thus the increase in NA content in the hearts of cold-acclimated rats also fits the concept of increased sympathetic nervous activity being able to induce the synthesis of increased stores of noradrenergic transmitter as discussed in Chapter 3 Section C. The fact that cold-acclimated rats did not show significant increase in the concentration of NA in the heart as was found in the trained rats could well be due to the physiological stress being of less severity although constant rather than intermittent in nature than the stress of exercise. It need not be a characteristic of cold-acclimation as such.

The finding of decreased NA content in the spleens of cold-acclimated rats is somewhat puzzling perhaps the increased peripheral vasoconstriction combined with high circulating levels of NA, partly of adrenal origin leads to decreased sympathetic nervous activity in the spleen. This suggestion is supported by the data of Jansky and Hart (1968) indicating that the blood flow to the spleen, in contrast to that of most other vascular beds, is the same in warm- as well as cold-acclimated rats when exposed to 30°C or to 9°C. Thus, the spleen seems not to be actively involved in the circulatory adjustments following acute or chronic exposure to cold.

The total NA content, and the relative proportion of NA, was significantly increased in the adrenal glands of cold-acclimated rats in agreement with the work of Kvaternicky *et al.* (1971). Several authors (see review by Himmels-Hagen, 1976) have reported an increase in the amount of adrenaline as well as that of NA in the adrenals of cold-acclimated rats, but although we also see a tendency towards increased adrenal adrenaline-content the difference is not significant. The increase in the adrenal NA content is likely to be mediated through induction of enzymes as discussed in Chapter 3 Section C, since the level of adrenal phenylethanolamine N-methyltransferase (PNMT) activity as well as the activities of tyrosine hydroxylase and dopamine  $\beta$ -hydroxylase are increased by cold stress, although both tyrosine hydroxylase and PNMT activity return to control values after 4 weeks cold exposure, when cold acclimation has occurred (Kvaternicky *et al.* 1971).

The observed increase in total NA content in the submandibular glands of cold-acclimated rats, as already discussed above is presumably also secondary to increased nervous activity in the sympathetic nerves.

#### Urinary excretion of catecholamines

Since chronically exercised rats excreted less NA during periods of cold stress than did sedentary warm-acclimated rats, and no more than the cold-acclimated rats excreted, the possibility arises that chronic exercise can also increase the sensitivity of tissues to the metabolic actions of catecholamines as occurs in cold-acclimated rats (see below). This may be due to the daily exposure of the tissues to higher than normal concentrations of catecholamines during exercise periods; thus, it is known that daily treatment of rats with NA sensitizes their tissues to the metabolic actions of NA (Le Blanc and Pouliot, 1964) although not to the cardiovascular actions (Le Blanc *et al.* 1969). The finding in adipose tissue of chronically exercised rats of an increased NA-sensitive lipolytic activity (Froberg *et al.* 1972) is consistent with the above suggestion. However, the increased sensitivity to NA in the vascular smooth muscle of chronically exercised rats (paper IV see Section G, Chapter 3) could also contribute to the observed lower increment in NA excretion in the cold by reducing transmitter demands for the maintenance of blood pressure and for the vasoconstrictor adjustments that occur during cold exposure (cf. Jansky and Hart, 1968).

In a study pertinent to the interpretation of the above findings, Strömme and Hemmet (1967)

report that although chronically exercised rats were able to increase their metabolic rate significantly more than control rats, and just as much as cold-acclimated rats, in response to cold stress and although they maintained higher peripheral skin temperatures during the cold exposure than control rats did, their metabolic response to injected NA nevertheless was the same as that of control rats. They therefore suggested that the enhanced capability of trained rats to elevate the metabolic rate during cold stress was mainly due to an "improved shivering capacity." Unfortunately there are several aspects of their experimental design that make their results difficult to interpret. Firstly they do not state whether the cold-acclimated rats were taken straight from their +5°C environment to the measurements of metabolic rate at various temperatures or whether they were allowed some time at room temperature in between. This is important since it is known that the metabolism of cold-acclimated rats remains elevated for at least 24 h after return to room temperature with a higher (than normal) urinary excretion of NA (Leduc, 1961) and, as will be discussed below, it probably takes at least 48 h for the metabolic rate of cold-acclimated rats to reach truly basal levels. In the study of Strömme and Hansson (1967) the metabolic rate of cold-acclimated rats at +26°C was almost 50% higher than that of controls suggesting that they had only very recently been removed from the cold environment. This among other things means that the measurements on the cold acclimated group do not start from a true resting level and also means that the blood levels of circulating catecholamines are higher in the cold-acclimated group than in the control group at the time when the various metabolic studies were made. Secondly the oxygen consumption is only measured from the 25th minute after the (subcutaneous) injection of NA and onwards, which means that they might have missed the peak metabolic response which occurs already 15 min. after the same dose given intramuscularly (Heath and Carlson 1957). Thirdly and probably less importantly the dose of NA given is calculated per kg BW which means that both the cold-acclimated and the trained rats received smaller doses of NA per animal. As the proportion of total body weight that is made up of "lean body mass" will be different in all three groups, the trained group having smaller fat deposits and more skeletal muscle mass, and the cold-acclimated group less skeletal muscle mass, than controls, it is virtually impossible to predict dose regimens which would give equal tissue levels of NA in all three groups. With these reservations in mind it is perhaps not surprising that not only do these authors fail to demonstrate an increased sensitivity to the metabolic effects of injected NA in exercised rats, they also failed to demonstrate the increased sensitivity to the catecholergic effects of NA characteristic of cold-acclimated rats. Admittedly the total metabolic response after the injection of NA was higher in the cold-acclimated group but this was entirely due to the fact that they started off from a 50% higher "resting" level, the net increase metabolic rate after the injection was of the same magnitude in all the experimental groups. This failure to demonstrate an enhanced sensitivity to the catecholergic effects of NA in the cold-acclimated rats, which clearly were acclimated to cold as shown by other results, suggests that their experimental design was not ideal.

Thus it appears that the question of whether or not chronically exercised rats develop increased sensitivity to the catecholergic effects of NA must be left open for the time being, although there are several indications that this may be the case.

The findings that cold-acclimated rats create not only significantly less NIA, but also less adrenaline, than control rats do during rest (at room temperature) as well as less NIA during cold stress, are seemingly in contrast to the results of Leduc (1961). Leduc found a higher urinary CA-excretion in cold-acclimated rats, when compared with warm-acclimated controls, during the first 24 h after return to room temperature while during the second day the CA-excretion did not differ from that of the controls; however Leduc did not collect urine after the second day. The urine collection in this study was made after the rats had been kept for 48 h at room temperature and thus our results indicate that it takes at least 48 h for cold-acclimated rats to reach a truly basal level of sympathetic activity after removal from the cold environment. The depressed levels of urinary CA-excretion in our cold-acclimated rats, when the animals are at room temperature, are probably mainly related to the



sensitivity to the calorogenic effects of both NA and adrenal ne which has been shown to occur in cold-acclimation (see Historical Background) and which presumably is also largely responsible for the smaller increase in the amount of NA excreted during cold stress in cold-acclimated animals than that found in controls, reported by Ladue (1961) and confirmed in this study.

The same net increase in the amount of NA excreted after exercise occurred in cold-acclimated rats as in the controls. The fact that there was a difference between the absolute amounts of NA excreted after exercise is because the cold-acclimated rats start from a lower basal level of NA excretion than the controls do. Thus it appears that cold-acclimated rats do not develop any "cross-tolerance" to the circulatory stress of exercise although chronically exercised rats do seem to develop a degree of cross-tolerance to the metabolic (and circulatory) stress of cold exposure. The reasons for this difference are not known. Both groups develop cardiac hypertrophy but conflicting results have been reported concerning the cardiovascular sensitivity to NA in cold-acclimated animals. Increased blood pressure responses to NA in cold-acclimated rats have been reported by Le Blanc (1960) and Herou (1961) on the contrary Honda *et al* (1962) found a decreased effect on vascular resistance. However these studies were performed in anesthetized animals with intact cardiovascular reflexes and probably animals just taken from a cold environment and thus with elevated blood levels of circulating catecholamines. Hsieh *et al* (1968) on the other hand using epinephrine, found slightly lower sensitivity to vasoconstrictor actions of NA at low dose levels, and no change at higher dose levels in cold-acclimated rats. Le Blanc *et al* (1972) also using anesthetized intact rats, reported a sensitization in cold-acclimated rats to the chronotropic, but not to the vascular effects of isoprenaline. Furthermore Hart *et al* (1974) reported that in the isolated atria of cold-exposed rats there is a decreased sensitivity to the chronotropic actions of NA after 7 days cold-exposure after 40-45 days of cold exposure they report that the dose-response curve for the chronotropic actions of NA is shifted to the left, but with a higher maximal response so that the ED<sub>50</sub>-dose remained unchanged; the significance of this observation is not clear however since they seem to lack age-matched control atria for this group. On the other hand Himmis-Hagen and Mazurkiewicz-Kwiecień (1970) also using isolated atria, found no change in the sensitivity to chronotropic and inotropic actions of NA in animals that had been acclimated to cold for 8-9 weeks.

Our observation that cold-acclimated rats responded with the same increment in urinary NA after exercise as did controls is more consistent with the reports suggesting an unchanged sensitivity to cardiovascular actions of NA, but other explanations cannot be excluded. Thus, another possibility is that the cold-acclimated laboratory rat is poorly adapted to muscular performance since these rats have a reduced mass of skeletal muscle (Herou and Gridgeman 1958) and since a characteristic of the circulatory adjustment to cold-acclimation is shift in distribution of blood flow from carcass and skin to internal organs and adipose tissues (Wienky and Hart, 1968) it is therefore conceivable although perhaps somewhat far-fetched that the increased "stress" of having to perform physical exercise with a smaller skeletal muscle mass would offset the decreased NA secretion from the cardiovascular system resulting from an increased sensitivity to cardiovascular actions of NA, and thus result in an unaltered total NA excretion after exercise. However the body weights of our cold-acclimated rats were not significantly below those of control rats and it is therefore by no means certain that these particular cold-acclimated rats did have a reduced muscle mass. In summary therefore it would appear most likely that the sensitivity to cardiovascular actions of NA was not increased in our cold-acclimated rats.

It may appear surprising that the vascular bed of cold-acclimated rats, exposed to elevated concentrations of NA and corticosterone (see Himmis-Hagen, 1976) should not develop an increased sensitivity to NA when that of the chronically trained rat does (see Section G Chapter 3). At least two factors could explain such a possible difference. Firstly cold exposure causes only modest increases in circulating NA levels as reflected by an about 3 to 3.6-fold increase in the urinary excretion (paper III) which is fairly evenly distributed over 24 hours apart from exercise on the other hand, causes peak

increase in circulating NA levels which can range from 8 to 80-fold (see review by von Euler 1974). It seems likely that the serum corticosterone levels might also show a similar pattern if one compares cold-acclimated rats to trained rats. Secondly the source of the NA overflowing into the circulation is probably to large extent different. In cold-acclimated rat considerable proportion of the NA is likely to come from increased sympathetic nervous activity in brown and white adipose tissue as well as the sympathetic nerves causing pilo-erection and vaso-constriction in the skin (see Jansky and Hart, 1968, and Himmle-Hagen 1975). In the exercising rat, on the other hand, a large proportion of the NA probably arises from increased sympathetic nervous activity in vasomotor nerves through activation of baroreceptor homeostatic mechanisms to prevent hypotension due to muscle vasodilatation (see reviews by Benavente and Shepherd, 1967 and von Euler 1974). Thus it seems very likely that the major part of the vascular bed of the trained rat will have been exposed to very much higher peak concentrations of NA than that of the cold-acclimated rat.

## CONCLUSIONS to Chapter 4

(1) Cold-acclimated rats show the same degree of cardiac and adrenal hypertrophy as trained rats but in addition also show significant hypertrophy of the submandibular salivary glands.

(2) Cold-acclimated rats show an increase in NA content in sympathetic nerve terminals in the heart and the submandibular glands. The NA concentration per g organ weight is however not significantly increased and the increase in cardiac NA content is not as great as that seen in trained rats.

(3) In room temperature environment, cold-acclimated rat excretes less NA in the urine than warm-acclimated control and the reduction in NA excretion is of the same magnitude as that seen in trained rats. However in contrast to trained rats, the cold-acclimated rat also shows reduction in the excretion of adrenaline in the urine in room-temperature environment. It is argued that this reduction in urinary excretion of catecholamines in cold-acclimated rats at rest reflects the increased sensitivity to the catabolic actions of catecholamines that characterize cold-acclimated animals.

(4) Following bout of exercise cold-acclimated rat excretes less NA in the urine than warm-acclimated untrained rat, but more than trained rat. In fact, the actual increment in NA excretion in the urine compared with the resting value is the same as that seen in warm-acclimated untrained rats but larger than that in trained rats. It is, therefore, suggested that cold-acclimated rats have no "cross-tolerance" to the largely circulatory strains of exercise and that they probably do not have the increased sensitivity to vasoconstrictor actions of NA the trained rats possess.

(5) During cold exposure the excretion of both NA and adrenaline in the urine is increased in all three groups however both cold-acclimated and trained rats show smaller rise in the urinary excretion of NA than that seen in warm-acclimated untrained rats. It is suggested that trained rats may display an increased sensitivity to the catabolic actions of NA similar to that seen in cold-acclimated rats and that regular physical exercise might confer some cross-tolerance to cold stress.

# STUDIES ON THE TURNOVER OF NORADRENALINE IN BRAIN AND HEART BASED ON SYNTHESIS INHIBITION BY $\alpha$ -METHYLTYROSINE

(previously unpublished data)

The fact that in steady state conditions the rate of synthesis of NA equals the turnover rate led Brodie *et al.* (1966) to suggest that the blockade of NA synthesis *in vivo* following administration of  $\alpha$ -methyltyrosine (Spector *et al.*, 1965) could provide a convenient method for the study of NA turnover by applying steady state kinetics and using the rate constant for the decline in endogenous NA levels as an estimate of the rate constant for NA turnover. This method for turnover estimation has become widely used because of the following advantages: 1) it is cheap, compared with isotope methods, and easy to use; 2) turnover in brain as well as in peripheral tissues can be studied; 3) both NA and DA turnover can be studied; 4) it avoids the pitfalls of the isotope methods in which there might be an unequal distribution of labelled amine between hypothetical readily available and "storage" pools. Accordingly, this method has been applied to study the turnover of catecholamines in the brain and in peripheral organs of rats trained by physical exercise.

## METHODS

After intravenous injection of  $\alpha$ -methyltyrosine the inhibition of NA synthesis is almost instantaneous (Brodie *et al.*, 1966).  $\alpha$ -methyltyrosine itself is however very poorly soluble and for that reason the methyl ester hydrochloride of DL- $\alpha$ -methyltyrosine (H 44/68, A.B. Heald) has become widely used. The methyl ester is rapidly converted *in vivo* to  $\alpha$ -methyltyrosine by an esterase (see Corradi and Hanson, 1965) and even after an i.p. injection the degree of inhibition of NA synthesis is more than 90% within 30 min (Parsons and Waldeck, 1970). After a single injection of 200 mg/kg BW the levels of  $\alpha$ -methyltyrosine in the rat brain remain high, well above inhibitory concentrations, for at least 8 h without any tendency to decline (Brodie *et al.*, 1966). In my experiments H 44/68 was given in an initial dose of 250 mg/kg BW i.p. (corresponding to 200 mg/kg of  $\alpha$ -methyltyrosine) followed by 125 mg/kg i.p. every 8 h in order to secure sufficiently high organ concentrations throughout one experiment. This dose regime has in fact been shown not only to ensure inhibitory tissue concentrations of  $\alpha$ -methyltyrosine throughout the experiment, but to lead to a continuous accumulation of  $\alpha$ -methyltyrosine in various tissues of the rat (Svedin 1970).

Male Sprague-Dawley rats initially weighing about 180 g. were divided in three groups, one of which was subjected to daily exercise in the form of swimming seven days per week, with the daily swimming period stepwise increased from 1 h to 2 h, during 14 weeks. A second group was acclimated to 4 °C and the third group served as controls. At the time of the turnover experiments their body weights were about 400–450 g. The turnover study was performed 48 h after the last period of exercise, or removal from the cold environment respectively. H 44/68 was given i.p., dissolved in saline in the doses described above. 12 rats were killed at 0 h (no H 44/68) and 3 rats each at 4 h, 8 h, 12 h and 16 h after the injection. Tissues were homogenized and catecholamines were separated from the extracts by chromatography as described in Chapter 2. NA was assayed according to Chung (1964) since assay according to Euler and Lishajko (1961) yields falsely high NA values, due to the fluorescence of  $\alpha$ -methyl DA and  $\alpha$ -methyl NA, when oxidation is carried out according to the latter method (Meire 1965).

## RESULTS

### General observations

There was a gradual onset of sedation, which became noticeable about 4 h after the injection and progressed to an almost "catatonic" state in which the rats huddled motionless with closed eyes, neither drinking nor eating. They were capable of moving a few steps when provoked but would not remove themselves from a strange environment, or show any exploratory behaviour and limbs placed in an unnatural position would not be moved for minutes. They also had a pronounced polyuria and appeared clinically dehydrated towards the end of the experiments (16 h).

### Body and organ weights

The trained rats exhibited the usual cardiac hypertrophy and reduction in body weight compared with the controls (see Chapter 3 Section III). Following the administration of H 44/68 there was a loss of body weight, due to dehydration, which had declined by 7% at 16 h. An organ with a normally high blood content such as the spleen displayed a 10% decrease in weight at 16 h whereas the weight of the brain remained unaffected throughout the experiment (see Fig. 6).

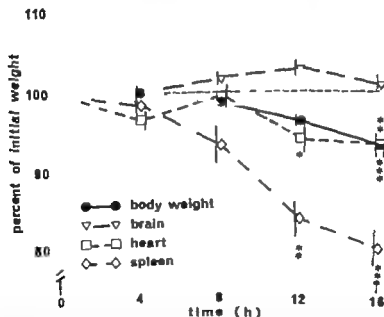


Fig. 5. The effect of  $\alpha$ -methyltyrosine-induced dehydration on body and organ weights. Doses of  $\alpha$ -methyltyrosine administered as described in the Methods section in this chapter. Each value is the mean of 12–14 observations and the vertical bars indicate standard error. \* denotes different from 0 h value  $p < 0.01$ ; \*\* denotes different from 0 h value  $p < 0.001$ .

### Norepinephrine turnover in brain and heart of trained rats (Fig. 6)

**Brain** The turnover of NA, as estimated by rate of decline in endogenous NA levels, is of the same magnitude as the seen in the  $^{14}\text{C}$ -tyrosine study ( $t_{1/2}$  about 5.5 h) only during the first four hours after synthesis inhibition (later called phase A) with  $t_{1/2}$  of 5.2 h in the control group and of 5.5 h in the

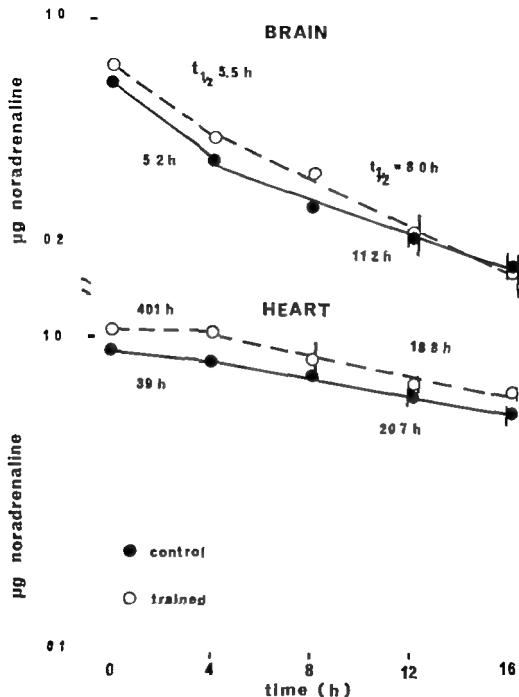


Fig. 6 Disappearance of noradrenaline in brain and heart of chronically exercised rats and of controls following treatment with  $\alpha$ -methyltyrosine. The values given are means  $\pm$  S.E., where vertical bars are absent S.E. is smaller than the circumference of the circle. The numbers in the left hand half of the figure are the calculated half-lives (in hours) for the disappearance of noradrenaline during phase A (0–4 h); those in the right hand half are the half-lives during phase B (4–16 h). For dose schedule see Methods section of this chapter.

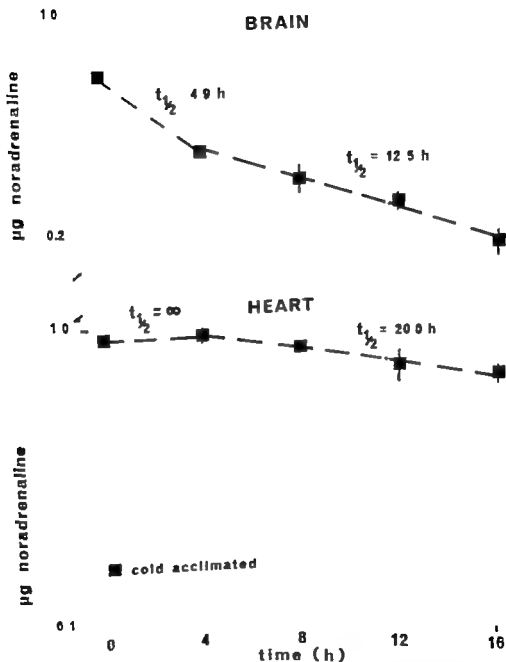


Fig. 7 Disappearance of noradrenaline in the brain and heart of cold-acclimated rats following treatment with  $\alpha$ -methyltyrosine.

The values given are mean  $\pm$  S.E., where vertical bars are absent, the S.E. falls within the area of the squares. The half-lives in the left hand half of the figure are the calculated half-lives for the disappearance of noradrenaline (in hours) during phase A (0–4 h) and those in the right hand half are the half-lives during phase B (4–16 h). For dose schedule see Methods section in this chapter.

trained group (n.s.) Subsequently during the 4 h - 16 h time interval (phase B) when the NA content of the brain has been depleted to 60% or less of the original level the fractional turnover rate (slope) is slowed down to give a  $t_{1/2}$  of 11.2 h in controls ( $p < 0.001$  v phase A) and to  $t_{1/2}$  of 8.0 h in the trained rats ( $p < 0.001$  phase A phase B control v phase B trained; n.s.)

**Hearts** Again during the first 4 hours the main findings (although not the exact values of the half lives) are the same as with the isotope methods (see Chapter 3 Section F). Thus there is significantly slower turnover of NA in the hearts of chronically exercised rats, in which the estimated  $t_{1/2}$  was 401 h compared with the hearts of controls, where the estimated  $t_{1/2}$  was 39 h ( $p < 0.05$  v trained). However during the 4 h - 16 h time interval (phase B) the turnover rate increases significantly in both groups, but more so in trained rats, so that during phase B the cardiac NA turnover is essentially the same in trained and untrained rats with  $t_{1/2}$  of 20.7 h ( $p < 0.02$  v phase A) for the control group and  $t_{1/2}$  of 18.8 h ( $p < 0.001$  phase A) for the trained group.

**Cold-acclimated rats.** That the above findings were not chance results is shown by the very similar appearance of turnover curves obtained by the same method in rats acclimated to cold as described in Chapter 4 (see Fig. 7). It is noteworthy that cold-acclimated rats, like trained rats show a non-significant turnover of norepinephrine in the heart in phase A, with  $t_{1/2}$  of infinity ( $p < 0.05$  controls), whereas in phase B the turnover rate accelerates, as in trained rats, to become indistinguishable from that of controls at a half life of 20 h.

## DISCUSSION

The results of the turnover studies performed by inhibition of NA synthesis are of interest in relation to the discussion in Chapter 7 concerning the possible inverse correlation between the activity of noreadrenergic neurons in the lower brain stem and the activity of peripheral sympathetic neurons, notably those innervating the heart. The regression lines for the decline in endogenous NA concentration versus time are distinctly biphasic in both brain and heart and it is noteworthy that the slowing of the turnover of NA in the brain occurs at the same time as the increase in cardiac NA turnover rate. It is very tempting to interpret these findings in terms of causal relation; in other words the decrease in sympathetic nervous activity seen for example in hearts of chronically exercised rats might be dependent on the increased activity of certain central noreadrenergic neurons and when the functions of these neurons are impaired, by loss of transmitter store or secondarily through involvement of other adrenergic pathways, the difference in peripheral sympathetic nervous activity between trained and untrained rats disappears. Before further discussion of this idea it must first be established that the biphasic curves are not artefacts due to experimental conditions.

### *Turnover of norepinephrine in the brain*

Insufficient synthesis inhibition by  $\alpha$ -methyltyrosine cannot explain the biphasic curve for the decline in NA content firstly because it is during the initial 4 h, when tissue concentrations of  $\alpha$ -methyltyrosine might have been suboptimal during the first 30 min, that the rapid turnover phase occurs and this is also the phase where the rate of NA turnover found is in good agreement with the turnover rate found in the  $^{14}$ C-tyrosine study and with turnover rates obtained by other workers (Iversen and Glowinski, 1966; Pearson 1969; Taylor and Laverly 1969; Nybeck and Sedvall 1970). Secondly in the 4-16 h interval when the turnover rate has slowed down, the tissue levels of  $\alpha$ -methyltyrosine are not only supra-optimal they are continuously increasing with the dose regime used in the present experiment (Sjweden 1970). Neither is it likely that this biphasic appearance is due to the





for instance a certain degree of depletion of transmitter stores. However the degree of curvilinearity in the 0-4 h interval is so small that the data given by these various authors are always fitted reasonably well by a straight line. For the sake of making a statistical comparison between the relative turnover rate of NA during phase A in different groups, it would thus seem to be justified to use an exponential regression line calculated by least square analysis as an approximation of whole brain turnover of NA even in the 0-4 h interval. The same argument also applies to phase A in the decline of NA levels in the heart since various authors have found the rate of decline during the 0-4 h interval to be exponential (Svedin 1970) or very close to exponential (Spector *et al.* 1966, Westfall 1970).

The biphasic appearance of the curve in all the above studies cannot be explained by insufficient drug concentrations in the brain since Brodie *et al.* (1966) found high concentrations of the drug in the brain and no tendency towards decrease, up to 8 h after a single injection of  $\alpha$ -methyltyrosine 200 mg/kg. Neither does the biphasic curve stem from the use of racemic as opposed to L- $\alpha$ -methyltyrosine since Porter *et al.* (1966) give data that, when converted to a conventional semi-logarithmic plot, show a biphasic decline in brain levels of NA in mice, both after administration of racemic or of L- $\alpha$ -methyltyrosine, again with a rapid phase between 0-4 h and a subsequent slower phase.

On the other hand, Nakamura *et al.* (1971) found undisputedly linear regression curves of NA content in the hypothalamus in the 0-9 h interval after administration of H 44/68. This could indicate that there are regional differences between the effect of synthesis inhibition by  $\alpha$ -methyltyrosine on the nervous activity of different noradrenergic pathways in the brain. Possibly age differences could also play a part in this discrepancy because whereas Nakamura *et al.* (1971<sup>2</sup>) use quite young rats most other workers use more adult animals.

It is noteworthy that there appears to be a correlation between the time at which the change from rapid to slower NA turnover occurs in the brain and the time at which some stress induced changes in the turnover of brain NA are abolished, just as there is in the alterations in cardiac NA turnover of trained rats found in my study. Thus the data of Corrodi *et al.* (1968) reveal that whereas the turnover of NA in the brains of rats subjected to continuous immobilization stress was increased in the 0-4 h interval it was the same as that of controls during the 4-8 h interval after H 44/68 administration, i.e. during the slower part of the "biphasic" curve (plotted from the results given by Corrodi *et al.* 1968).

#### *Turnover of noradrenaline in the heart*

Turning next to consider the findings in the heart, there are again several publications with data on the decline of endogenous NA content versus time where the results are much better fitted by a curve that is at least biphasic than by a single exponential regression line. In contrast to the curves in brains, however, the initial phase of NA turnover in the heart is the slow phase and the later phase is the one with more rapid rate of decline. Such data are given for the control animals e.g. by Spector *et al.* (1966) using guinea pigs and by Westfall (1970), Svedin (1970) and Mueller and Thoenen (1971) using rats. Common to all these studies, as well as to the curves in Figs. 6 and 7 is the fact that the acceleration of the turnover rate occurs 4-8 h after the onset of synthesis inhibition - in other words at the same time as the slowing of NA turnover in the brain occurs. Svedin (1970) speculated that the acceleration of NA depletion occurring after 16 h might be due to the formation of  $\alpha$ -methylated amines with a direct releasing effect upon endogenous NA stores. However Spector *et al.* (1966) were unable to detect formation of  $\alpha$ -methyltyramine in guinea pig brain and heart up to 8 h after the injection of the  $\alpha$ -methyltyrosine and thus this explanation cannot explain the acceleration of the turnover of NA seen after 4 h in their experiments. Furthermore Mäkinen (1968) showed that the amount of  $\alpha$ -methylNA and  $\alpha$ -methylDA, neither of which have direct releasing effect on endogenous NA stores, formed 16 h after administration of  $\alpha$ -methyltyrosine is much higher in the brain than in the

heart. One might then expect that formation of  $\alpha$ -methylated metabolites with direct releasing effects, such as e.g.  $\alpha$ -methyltyramine, would also be greater in the brain than in the heart. However in the brain no acceleration of turnover rate but rather the opposite occurs with increasing time. Thus it appears that at least during the time interval studied, 0–16 h the observed acceleration of the rate of decline of the NA content of the heart from about 4 h onwards cannot be accounted for by the releasing action of  $\alpha$ -methylated metabolites.

There are however many publications where the regression curves of NA versus time in the hearts of both rats and mice appear to decline exponentially after synthesis inhibition by  $\alpha$ -methyltyrosine i.e. with data that well fit a single regression line in a semilogarithmic plot (Porter *et al* 1966 de Champlain *et al* 1969 Bralet *et al* 1971 Nakamura *et al* 1971 and <sup>b</sup> and Lemmer and Saller 1974). It is noteworthy that all these authors base their regression curves on the decline in NA concentration ( $\mu\text{g/g}$ ) in heart rather than on decline in total NA content in the heart. This way of expressing their results introduces a quantitatively important artefact since during the experiment progressive dehydration occurs secondary to the polyuria resulting from the kidney toxicity of  $\alpha$ -methyltyrosine (Moore *et al* 1967). With increasing dehydration the weights of most organs including the heart, but excluding the brain, decreases (see Fig. 3) and consequently falsely high values (in relation to dry weight) will be obtained when the NA levels in an organ are expressed as  $\mu\text{g NA/g}$ . If this progressive dehydration led to an exponential decline in organ weight this experimental artefact would, depending on the half-life, not necessarily distort the regression line of NA ( $\mu\text{g/g}$ ) versus time but could simply give falsely low values of the slope leading to an erroneously high estimate of half-life. However, as seen in Fig. 5 the decline in body weight is not exponential but starts slowly in the 4–8 h interval and accelerates after 8 h. The decrease in heart weight parallels that in body weight fairly closely although in the first 8 h the change in heart weight lags behind that in body weight (see Fig. 6). If the decline in cardiac NA expressed as  $\mu\text{g/organ}$  was exponential, then the superimposed dehydration occurring during the later part of the experiment would artefactually lead to an apparent slowing of the turnover rate when the data are expressed as  $\mu\text{g NA/g wet weight}$ . That this slowing is not observed by the authors who base their estimations of turnover rate on NA concentration in the heart rather than total NA content is in fact an indirect indication that there was in fact an acceleration of the total NA turnover in the later part of their experiment. Most of these authors (de Champlain *et al* 1969 Bralet *et al* 1971 Nakamura *et al* 1971 and <sup>b</sup> and Lemmer and Saller 1974) used a dosing schedule of  $\alpha$ -methyltyrosine which would lead to a more rapid build up to high tissue levels of the drug than the dosing schedule used by Sweden (1970) and myself and thus presumably also to an earlier onset of tubular necrosis with ensuing polyuria and dehydration. Thus it is quite possible that in these experiments the onset of dehydration leading to decreased heart weight coincided in time with the acceleration in cardiac NA turnover that was seen after 4–8 h in my work (Figs. 6 and 7) and in the studies, discussed above, of Spector *et al* (1966) Wiertz (1970) Sweden (1970) and Mueller and Thoenen (1971). As a result, the change in heart weight would have masked the increased turnover of NA and so would have led the group of authors who expressed their results in terms of NA concentration to underestimate the true rate of turnover. On any account, it is not justified for this group of workers to ignore such changes in organ weights (in heart –8% at 12 h and –7% at 16 h and in spleen –16% at 12 h and –19% at 16 h) in their studies on transmitter turnover. This objection does not apply to the brain, however, since the weight of the brain remains unaltered at least in the 0–16 h time interval.

It is interesting to consider in more detail the experiment of Mueller and Thoenen (1971) on the effect of aprepidine on NA turnover in the rat heart. Their data for hearts from controls (expressed in  $\mu\text{g NA/hearts}$ ) indicate a clearly biphasic decline in NA levels after inhibition of NA synthesis by  $\alpha$ -methyltyrosine. The faster decline in cardiac NA levels in aprepidine-treated rats, on the other hand, seems to be truly exponential. Thus appears that where the rate of turnover of NA in the heart is already high, and the turnover of NA in the brain stem probably lower than normal, no further

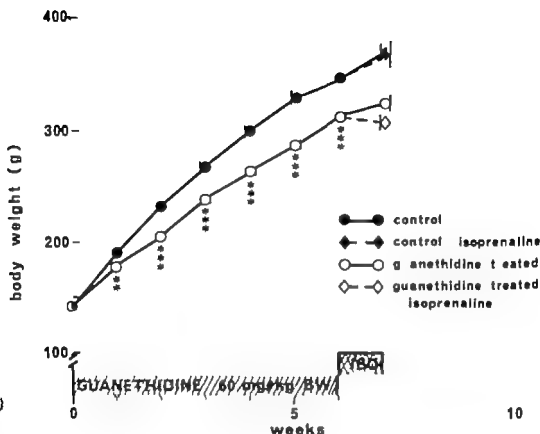
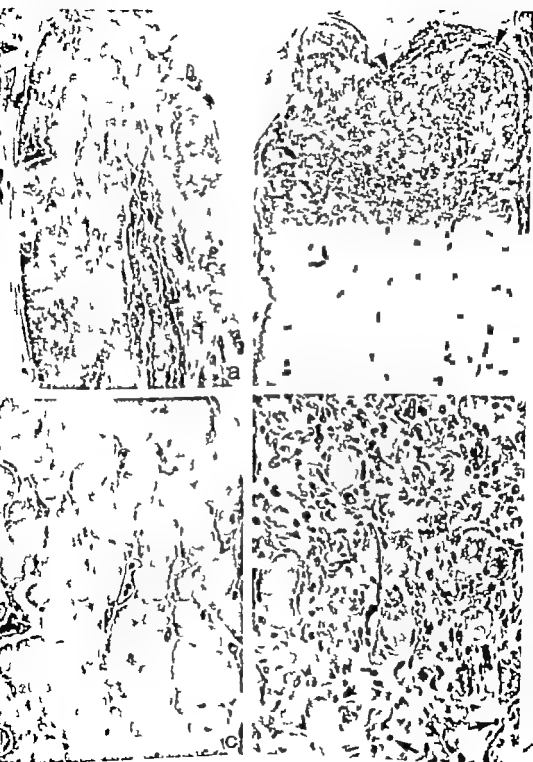


Fig. 8. Growth curve of guanine and isoprenaline treated male Wistar rats.

Values given are means and vertical bars denote  $\pm$  S.E. Where vertical bars are absent the S.E. falls within the area of the symbol. The striped bar along the x-axis shows the duration of daily treatment with guanine (60 mg/kg BW daily i.p.) and the cross-hatched bar shows the duration of treatment with isoprenaline (0.2 mg base/kg BW i.v. twice daily s.c.) denotes different from control,  $p < 0.01$ ; denotes different from control,  $p < 0.001$ .

Fig. 9. Scellate ganglia of control and guanine-treated (60 mg/kg BW/day i.p. for 5 weeks) rats. 1–2  $\mu$ m Araldite sections of ganglia removed post mortem and fixed by immersion in cacodylate-buffered 1% osmium tetroxide pH 7.2. Stain: Methylene blue and Azur II. a, b. General view of part of each ganglion X100. c, d. Views at higher magnification (X250) of areas taken from a and b. The control ganglion (a, c) has compact structure and is closely packed with neurons and with bundles of nerve fibres with their satellite and Schwann cells. The guanine-treated ganglion (b, d) is not smaller than the normal ganglion but has much looser texture; few neurons are seen but almost all of these look normal and are at least as large as the normal neurons. A few are smaller and have displaced nuclei and poorly defined Nissl material. The intervals between the surviving neurons contain rather irregular bundles of nerve fibres and Schwann cells in an expanded intraganglionic tissue space which shows no light infiltration by lymphocytes (small round cells, arrows, d) and a few mono-nuclear cells. At the periphery of the ganglion in one place (b, between arrowheads, upper right) the capsular tissue is rather densely infiltrated by small round cells. Such localized heavier accumulations of lymphocytes were also sporadically distributed in all the sympathetic ganglia from the guanine-treated rats. (cf Fig. 2 of Burnstock et al. 1971). Previously unpublished results (M. R. Matthews and I. Ostman-Smith).





### Experiments on rats treated with isoprenaline

*Effect of isoprenaline-treatment per se* (see Table 4) The isoprenaline-treatment given did not significantly alter the body weight or the weight of the submandibular gland in non-sympathectomized rats. In the short time of seven days it did however cause an impressive cardiac hypertrophy with 24% increase in heart wet weight. This was accompanied by a probable slight decrease in the proportion of cardiac weight constituted by dry matter as shown by the dry weight percentage (see Table 4). Somewhat unexpected was the finding that the kidney wet weight was 12% lower in the isoprenaline-treated animals ( $p < 0.05$ ); furthermore when the effect of individual variations in body size was diminished by expressing the data as "kidney ratio" (g/100 g BW) the decrease is statistically even more significant ( $p < 0.01$ ).

*Effect of guanethidine treatment (50 mg/kg BW) per se* (see Figs. 8 and 9 Table 4) In the higher dosage employed in these experiments the effect of the guanethidine-treatment on body growth was more pronounced than in paper VI, already two weeks after starting the drug treatment the body weights of the guanethidine treated rats were 12% lower than those of untreated rats, and the relative difference remained at that level throughout the experiment, with the final body weight of the guanethidine treated controls (group C) 13% lower than that of untreated controls (group A). Body growth as reflected by body length is also significantly decreased at this dose schedule: this can be seen if the data from both guanethidine treated groups are pooled and compared with data from both untreated groups, the pooled mean body length (exclusive of tail) of guanethidine treated rats being  $23.9 \pm 0.2$  cm, 3% less than that of the untreated group which was  $27.7 \pm 0.2$  cm ( $p < 0.01$ ). In this experiment the impairment of growth involved most organs to about the same degree as it did whole body growth: expressed by body weight and so although the weights of the heart (-15%), the kidney (-12%) and the submandibular glands (-18%) were significantly lower when compared with those of these organs from untreated controls (group A) the heart ratio, kidney ratio and submandibular gland ratio (organ weight in g/100 g BW) were not significantly altered. Likewise cardiac dry weight as per centage of wet weight was not significantly altered.

General observations were the same as quoted above from paper VI including the impression of smaller fat deposits.

There was no detectable tyrosine hydroxylase activity in superior cervical ganglia, which with the sensitivity of the method, means that tyrosine hydroxylase activity was decreased by at least 80%. The profound loss of sympathetic ganglion cells in splanchnic ganglia of the guanethidine-treated group is shown in Fig. 9, and in superior cervical ganglia there were 1103 neurons/ganglion compared with 32,546 in control rats (M.R. Matthews and M.E. Flacey personal communication).

*Effect of guanethidine-treatment on the isoprenaline response* Whereas non-sympathectomized rats withstood the isoprenaline treatment well with no obvious symptoms and no fatalities, the guanethidine-treated rats subjectively were more affected and some animals would lie prostrate on the floor of the cage although they were able to get up and move when disturbed. An increased sedation was not infrequently noted in guanethidine-treated rats following isoprenaline administration. As mentioned above there were several fatalities due to isoprenaline treatment in the guanethidine-treated groups, the majority occurring within one to two hours after the injection but a few occurring many hours later. Those deaths actually witnessed were undoubtedly sudden deaths, probably due to cardiac arrhythmias, and one such rat was actually successfully resuscitated with external cardiac massage.

As clearly demonstrated in Fig. 8, the isoprenaline treatment had a marked depressing effect on the rate of weight gain in the guanethidine treated rats, causing them to completely stop gaining weight during the isoprenaline treatment: this is in contrast to the findings in the non-sympathectomized

The isoprenaline-induced cardiac hypertrophy was not prevented by the chemical sympathectomy. Isoprenaline treatment caused a 31% increase in the heart weight of guanethidine-treated rats compared with a 24% increase in controls. There was a tendency for decreased kidney weight in isoprenaline-treated sympathectomized rats (-11% when compared with the guanethidine only group) but this was not statistically significant.

## DISCUSSION

### *Chemical sympathectomy with guanethidine*

**Efficacy of sympathectomy** The first question that must be considered is the degree of completeness of the sympathetic denervation, particularly in the cardiovascular system which is the most pertinent for the interpretation of the results in this study. The results of the NA estimations in the various organs indicate that the loss of sympathetic innervation is extensive but not complete. On the surface it may seem difficult to reconcile findings of remaining NA levels varying between 10 to 17% with the assessment by Burnstock *et al.* (1971) that less than 2% of the sympathetic ganglion cells remain intact after the same treatment schedule using rats of the same strain, age and sex; however Heston *et al.* (1972)

observed that the few sympathetic ganglion cells that remained were hypertrophied and appeared to give rise to a larger number of processes than normal. Furthermore Blythe *et al.* (1976) also using the same treatment schedule in Sprague-Dawley rats, reported that in contrast to the findings of Burnstock *et al.* (1971) they did see some evidence of regeneration of sympathetic nerve terminals, as indicated both by fluorescence histochemistry and NA assays. Thus they reported that whereas there were no detectable adrenergic nerve terminals in the ventral caudal artery 24 h after cessation of the guanethidine treatment, already after two weeks occasional fluorescent terminals were seen in half of the specimens and after ten weeks 11 specimens examined showed an adrenergic vascular innervation,

although still attenuated compared with controls. In the heart there was no detectable NA one week after cessation of guanethidine treatment, but after two weeks the NA level had increased to 9% of normal and after eight weeks the cardiac NA content was 22% of normal. Although at the same time only rare adrenergic fibers, few of which were intensely fluorescent, were found on fluorescence histochemistry in the atria (Blythe *et al.* 1976). It is likely that the pattern of reinnervation after chemical sympathectomy with guanethidine treatment is the same as that seen after 6-OHDA treatment, in which case the reinnervation of the atrium occurs earlier and is much more complete than the reinnervation of the ventricles which remains largely restricted to vascular innervation (Jacobowitz 1975). The association of only rare fluorescent fibers, as seen on fluorescence histochemistry in atria,

with cardiac NA content of 22% of normal in the study of Blythe *et al.* (1976) might perhaps be explained in terms of piling up of neurotransmitter in regenerating non-terminal zones similar to that seen after ligation (Dehlstrom, 1966) and after 6-OHDA treatment (Malmfors and Sachs, 1968). Considering the above observations it appears that in the present study the denervation of the heart has been complete for a minimum duration of half of the experimental period, and that a reinnervation that occurred in the heart during the second half was small. Cardiac NA of the experiment reaching 17% of normal and probably largely confined to the reinnervation of the vasculature and to a small extent of the atria while the ventricular myocardium is had any significant degree of reinnervation.

Considering the vascular bed, reports are again somewhat conflicting. Only occasional fluorescent fibers even at four months after cessation of treatment, (1976) find a regular but sparse reinnervation after 10 weeks (see above). From our view Blythe *et al.* (1976) reported that pressor responses to aortic occlusion were still present after chronic guanethidine treatment, although reduced in amplitude.

adrenal medulla was left intact, however and the adrenal medulla may contribute to the pressor response after both these treatments (Heymans and Neil 1958; Lalonde *et al.* 1968). Blythe *et al.* (1978) did in fact also demonstrate that there was considerable degree of denervation supersensitivity in the vasopressor response to L.V. NA in their guanethidine-treated rats 10 weeks after cessation of treatment, with six-fold increase in sensitivity at low doses and two-fold increase in sensitivity at high doses of NA, the altered slope of the dose-response curve suggesting abolition of neuronal re-uptake (see Truedssonberg, 1972).

It seems likely therefore that small degree of vascular reinnervation may occur but that within the time period involved in the present study the reinnervation is far from complete and denervation supersensitivity of vascular smooth muscle persists. However Westfall *et al* (1975) have reported that in the denervated vas deferens the number of neural contacts between individual smooth muscle cells increased, and if the same is true also for vascular smooth muscle small degree of reinnervation may be not inconsiderable functional significance. This is in agreement with the findings of N.O. Sjostrand and T. Sjostrand (personal communication) that in guanethidine-treated rats the carotid occlusion reflex persisted even after denervation of the dorsal medulla.

influence of guanethidine treatment on body and organ growth. Decreased weight gain has been reported in rats treated with guanethidine (Eränkö and Eränkö, 1971; Johnson et al 1975) but these authors do not comment on the size of individual organs and no data seems to have been published previously on the effect of chronic guanethidine treatment on body and organ growth in young adult rats. The effect on weight gain may be roughly dose-dependent since treatment with 20 mg/kg BW leads to 7% lowering of body weight (paper VI) 60 mg/kg 13% lowering of body weight (present study) and 100 mg/kg in newborn rats lowers adult body weight by 20-30% (Johnson et al 1975). The effect is not related to the immediate effects of guanethidine as such though since it persists after discontinuation of the treatment (refs. as above) and it is not related to non-specific effects of intraperitoneal injections since it is seen also after subcutaneous administration (Johnson et al 1975). It is possible that the age at the start of treatment is an influencing factor since the most pronounced weight depression was reported in neonatally treated rats (Johnson et al 1975) the least in rats treated with rats weighing about 140 g (age 8 weeks) at the onset of treatment (present study) and the smallest effect on rats weighing about 180 g (age 7 weeks) at the onset of treatment (paper VII). Several observations suggest that the impairment of weight gain is related to the degree of symp-  
nectomy. Firstly oral treatment with high doses of guanethidine (about 100 mg/kg BW/day for 2-3 weeks fails to achieve significant symp-  
nectomy and also fails to impair weight gain (L. Chubb and A.D. Smith unpublished observation). Secondly long term treatment of rats with the  $\beta$ -blocking agents piroxolol or propranolol has been reported to result in decreased body weight (Vaughan Williams and Raine, 1974; Vaughan Williams et al 1975). Immunoregulation of the other hand has not been reported to interfere with body growth (see Linn-Montell and Engström, 1966; Clark 1971) but this may be related to the fact that the degree of symp-  
nectomy is poor (Iversen et al 1968; Thoenen 1972) the gastro-intestinal tract, including the liver is poor (Iversen et al 1968; Thoenen 1972) the possible site of action of the growth inhibiting effect of the

Any suggestions as to the possible site of action of this growth inhibiting effect of guanethidine sympathetomy by guanethidine must be purely speculative. It is unlikely that the effect of guanethidine on stool consistency, no really amounting to diarrhoea, would reflect gastroenteric motility changes enough in itself to impair absorption sufficiently to cause malnutrition. As the normal mucosa itself is no normally innervated by adrenergic nerve terminals also appears under the influence of nervation would influence absorption via interfering with the normal function of intestinal mucosa (see review by Holdsworth and Sherman, 1972). The effects of MA on various organ systems such as carbohydrate and lipid metabolism and on protein synthesis such as liver protein are well known and adipose tissue are complete (see review by Himmle-Hagen 1972) and some others as well.



apparent explanation as to why chemical sympathectomy should cause partial inhibition of growth, particularly since the noreadrenergic innervation in the liver is presumed to be largely vascular and not in the parenchyma (see review by Holtzauer and Sherman, 1972)

When the effect of chemical sympathectomy on the growth of individual organs is considered a quantitative difference between the results in paper VI and the results in the subsequent study using a higher guanethidine dose becomes apparent. In the first study where the retardation of whole body growth is mild and body length not significantly altered, the weight of the heart remains the same as in control rats of the same age and consequently the heart ratio is increased in guanethidine treated rats. In the second study where the inhibition of growth was more pronounced and body length was also significantly lower than in controls the heart size was decreased in proportion to the lowering of whole body weight, in other words the heart ratio remained unaltered. This difference could conceivably be due to strain differences in the reaction to chemical sympathectomy by guanethidine, since Sprague-Dawley rats were used in the first study and Wistar rats in the second. However it is also possible that this could be explained by the existence of a critical level of impairment of anabolic activity presumably through lack of amino acids and energy sources, so that if the supply of "building material" is only slightly reduced normal growth of vital organs continues at the expense of for instance adipose tissue whereas if the supply is more markedly depressed all anabolic activity is impaired to a roughly equal degree in the absence of preferential demands on organ function. Such an hypothesis is compatible with the observation that if animals are submitted to starvation or marked undernutrition heart weight and body weight decrease in parallel (Jackson, 1926 Vs Liens and Steeth 1936; Bezdek, 1954) whereas when obese rats are submitted to food restriction with an adequate protein intake body weight decreases more than heart weight, resulting in an increased heart ratio (Oscal and Holloszy 1970). If the obese rats in the latter study were exercised they lost body weight to the same extent but without any loss of heart weight, thus demand on organ function appears to play a role in determining which tissues hypotrophy (Oscal and Holloszy 1970)

Surgical denervation of salivary glands has been reported to cause a decrease in weight of the gland (Wells *et al* 1961) but in both my studies with chemical sympathectomy by guanethidine treatment the decrease in weight of the submandibular gland was proportional to the decrease in whole body weight and thus this may be a non-specific finding. The same is also true for the lowering of kidney weight found in the second guanethidine study

**Urinary excretion of catecholamines.** The finding that the urinary excretion of NA remains about half of that seen in control animals is in agreement with what has previously been found in immunosympathectomized rats (for references see above). A proportion of the NA excreted in the urine may derive from degree of reinnervation of the vasculature but most is likely to derive from compensatory increase in adrenal medullary secretory activity. This assumption is based on the following observations: (i) the adrenal content of NA is increased to a significantly higher degree than the content of adrenaline (ii) the blood pressure of rats chemically sympathectomized with 6-OHDA falls drastically after adrenalectomy (with steroid substitution) or even just clamping the adrenal vein (de Champlain and van Ameringen, 1973) (iii) immunosympathectomy increases the rate of catecholamine turnover in the adrenal medulla (Iversen *et al* 1968) (iv) chemical sympathectomy with 6-OHDA increases the tyrosine hydroxylase activity in the adrenal gland (Muster *et al* 1969). Since the urinary excretion of adrenaline remained unchanged in the guanethidine treated rats, it appears that the increased secretory activity of the adrenal medulla is selective and mainly involves NA containing cells. Such a mechanism is compatible with the observations that the NA/adrenaline ratio in adrenal venous blood is different after different experimental stimuli of adrenal medullary secretion (see review by Lewis 1975) and that hypothalamic stimulation of different areas can induce preferential secretion of one or other of the catecholamines (Folkow and von Euler 1954)

The compensatory adrenal medullary release of NA, together with the denervation supersensitivity of the vascular smooth muscle to NA, and probably some degree of reinnervation of the vasculature and in addition the intact renin-angiotensin system, provide compensatory mechanisms which keep peripheral resistance and blood pressure normal (see de Champlain *et al.* 1975). Le Blanc *et al.* (1969) report normal blood pressure in guanethidine-treated rats (after treatment leading to denervation supersensitivity) and N.O. Sjöstrand and T. Sjöstrand (personal communication) found that under anaesthesia the blood pressure of guanethidine-treated rats ( $130 \pm 1$  S.E., mm Hg) did not differ from that of controls ( $128 \pm 8$  mm Hg). Blythe *et al.* (1978) state that the blood pressure of guanethidine-treated rats is lowered but they recorded blood pressure under anaesthesia in the carotid artery which introducing a one-sided carotid-occlusion, normally elevates the blood pressure by about 25–35 mm Hg (Folkow *et al.* 1970). The blood pressure level they report for guanethidine-treated rats (mean blood pressure ranging between 125–140 mm Hg) is in fact clearly above normal and the blood pressure of the controls, mean blood pressure about 155 mm Hg, is distinctly hypertensive. As they showed in the same study that the pressor response to carotid occlusion was decreased in guanethidine-treated animals there is no evidence that the resting blood pressure (i.e. before tying off one carotid artery) in their guanethidine-treated rats was lower than normal.

There was a very constant relationship between the urinary excretion of NA of guanethidine-treated rats and that of untreated rats, both during rest (46% of that of the untreated) and after exercise (40%) which means that the guanethidine-treated rats were able to increase the amount of NA released into the circulation when necessary. It appears therefore that the amount of NA excreted in the urine at rest reflects the minimal amount of circulating NA needed to maintain homeostasis. Thus the degree of reduction in NA excretion seen at rest in the guanethidine-treated rats is probably largely determined by the extent of denervation supersensitivity of vascular smooth muscle. It is noteworthy that chemical sympathectomy by inducing denervation supersensitivity abolishes the normal differential between sedentary and trained rats (with lower urinary excretion of NA in chronically exercised rats; see Section D). This is further indication that the exercise-induced lower level of NA excretion is linked to the increased sensitivity to NA obtained in vascular smooth muscle of trained rats (see Section G, Chapter 3).

**Effect of exercise on chemical sympathectomy with guanethidine.** Chronic physical exercise normally produces an increase in cardiac NA content and possible mechanisms of this increase are discussed elsewhere (see Section C, Chapter 3). It is noteworthy that the residual cardiac NA content after chemical sympathectomy with guanethidine was twice as high in the group that was submitted to daily exercise as in the sedentary group. There are several possible explanations for this observation. Firstly it is possible that each individual surviving cardiac noradrenergic neuron is induced by the exercise to increase its NA stores as occurs in the non-sympathectomized animal. Secondly it could be due to increased stores of NA in the cardiac intramural small fluorescent cells that have been described to occur in rats and are commonly called chromaffin cells although they do not always exhibit positive chromaffin reaction (Jacobowitz 1967). The second explanation appears unlikely however as the fluorescent substance in these cells has not been shown to be NA and it has been suggested that it is dopamine (Ehlfors *et al.* 1968). Furthermore cardiac denervation by autotransplantation which still leaves the cardiac chromaffin cells intact (Jacobowitz 1967) reduces cardiac NA content to undetectable amounts (Jacobowitz *et al.* 1967). Thirdly it is possible that the exercise-induced increase in cardiac sympathetic nervous activity leads to decreased susceptibility of the noradrenergic neuron to the toxic effects of the guanethidine treatment as it has been observed that neurons with low degree of sympathetic tone such as the short noradrenergic neurons of the vas deferens are chemically denervated at dose level (5 mg/kg BW/day i.p.) which does not produce denervation of the rest of the sympathetic nervous system (Gannon *et al.* 1971) and, furthermore, denervation of the superior

cervical ganglion results in increased sensitivity to guanethidine-induced CA depletion (Heath *et al.*, 1972). However in the spleen, another organ that takes part in the vascular readjustment during exercise the NA depletion after guanethidine treatment is if anything more pronounced in exercised animals. Fourthly as the noradrenergic terminals to the vasculature are the first to regenerate (see above) the increased NA content of the heart may be related to increased vascularization which usually follows chronic exercise (reference see Section C); it has not been established however whether vascular neoformation in response to chronic exercise still occurs in the absence of adaptive cardiac hypertrophy.

#### *Effect of isoprenaline treatment*

In the dose schedule employed in the present study the effect of isoprenaline treatment on the non-sympathectomized animal was restricted to considerable degree of cardiac hypertrophy (+24%) and significant decrease in kidney weight (-12%). Generalized cardiac hypertrophy involving both sides of the heart (see Albert, 1971) following isoprenaline treatment was first reported by Stanton *et al.* (1969) and this finding has subsequently been confirmed by several authors (Le Blanc *et al.* 1972 and others, see Cohen 1974 for references). Very large doses of isoprenaline (80 mg/kg BW) cause severe gross cardiac necrosis (Rone *et al.* 1959; Stanton *et al.* 1969) presumed to be due to a simultaneous marked increase in metabolic demands and the low perfusion pressure (Stanton *et al.* 1969). More moderate doses, about 5 mg/kg BW cause not macroscopical infarcts but microscopic lesions with histiocytosis, myocardial cell oedema as well as truly hypertrophied myocardial cells with neoformations of myofibrils are seen (Stanton *et al.* 1969; Pfitzer *et al.*, 1972) and the end result is cardiac hypertrophy with an element of fibrosis (Stanton *et al.* 1969; Bartosova *et al.* 1969). However low doses of isoprenaline 0.1 mg/kg BW daily - 0.3 mg/kg BW twice daily also produce cardiac hypertrophy (Stanton *et al.* 1969; Le Blanc *et al.* 1972; Cohen 1974) without causing any cellular damage detectable by light and electron microscopy (Cohen 1974). The significance of the ability of isoprenaline to induce cardiac hypertrophy will be discussed in greater detail below.

The etiology of the observed decrease in kidney weight is obscure unless the rapid hypertrophy of the heart actually provokes catabolism of other tissues which would be somewhat surprising as the non-sympathectomized rats continued to grow normally during the isoprenaline treatment.

Large systemic doses of isoprenaline 20-80 mg/kg BW twice daily (doses which carry considerable mortality) cause marked hypertrophy and hyperplasia of submandibular and parotid salivary glands, but not of sublingual and lacrimal glands (see review by Himms-Hagen, 1972). This effect is blocked by  $\beta$ -blocking agents (Campos and Parr 1968) and is not prevented by pretreatment with reserpine (Potito, 1966) or surgical sympathectomy (Campos and Parr 1968) thus it is presumed to be due to a direct  $\beta$ -agonist effect on the salivary gland. It is noteworthy that isoprenaline as well as NA stimulates secretion of amylase from the salivary gland (see review by Himms-Hagen, 1972). In this study salivary gland hypertrophy did not occur in non-sympathectomized rats after short term treatment of isoprenaline in doses of 0.2 mg/kg BW doses which nevertheless had marked cardiovascular effects and results in considerable degree of cardiac hypertrophy in the guanethidine-treated rats; however there was significant increase in submandibular gland weight following isoprenaline treatment. This confirms the observations of Campos and Parr (1968) that isoprenaline-induced salivary gland hypertrophy is not inhibited by denervation and furthermore it raises the possibility that a denervation supersensitivity may occur for the trophic responses of the salivary gland as well as for secretomotor responses.

#### *Role of the sympathetic nervous system in exercise-induced cardiac hypertrophy*

There are two possible explanations for the observation (paper VI) that chemical sympathectomy

by guanethidine-treatment completely abolishes the compensatory cardiac hypertrophy induced by physical training.

(ii) That an increase in work-load is in itself sufficient to evoke cardiac hypertrophy but that the hearts of the guanethidine-treated exercised rats were not subjected to any significant increase in work load.

(iii) The cardiac sympathetic nerves normally release a substance which is necessary for the development of exercise-induced adaptive cardiac hypertrophy.

There are several arguments against the first explanation. Not only were the guanethidine-treated rats subjected to the same swimming schedule as the untreated-exercised group they also displayed the same spontaneous swimming activity. Furthermore the effectiveness of the exercise is revealed by the fact that the mean body weight of each exercised group was reduced by exactly the same proportion, when compared with that of the respective sedentary control group.

It could be argued that chemical sympathectomy might cause a profound drop in peripheral vascular resistance leading to a decreased work load for the heart. However several compensatory mechanisms including increased adrenal medullary secretory activity and an activation of the renin-angiotensin and aldosterone systems combine to restore the blood pressure (see Introduction); and although some but not all workers who have measured the blood pressure of immunosympathectomized rats under anaesthesia find slightly subnormal blood pressure (possibly due to impaired ability of the immunosympathectomized rats to compensate for the hypotensive action of anaesthesia) if studies performed on conscious immunosympathectomized rats their blood pressure was found to be normal (see Introduction). If one takes into consideration the degree of denervation supersensitivity to NA in the vascular bed of sympathectomized rats, which is of the order of two-to-six-fold increase in the sensitivity in guanethidine-treated rats (Blythe *et al.* 1976) then the level of circulating NA in the sensitivity in guanethidine-treated rats (as revealed by urinary excretion of NA that is only 45-46% lower than that of controls) is likely to be sufficient to maintain a normal peripheral vascular resistance both at rest and during exercise. This is in agreement with the finding of Blythe *et al.* (1976) that rats subjected to chemical sympathectomy with guanethidine treatment were not hypotensive and with the unpublished observations by N.O. Sjöstrand and T. Sjöstrand (see above) that the blood pressures of such rats are no different from those of control rats when measured by intra-arterial recording under anaesthesia.

Furthermore if chronic guanethidine treatment did lead to a significant permanent drop in peripheral resistance leading to a decreased work-load for the heart, one would expect, on the work-load hypothesis, that the guanethidine-sedentary rats would have significantly smaller hearts than the untreated-sedentary group; however the heart weights in these two groups were found to be the same (in paper VI).

It could also be argued that the denervated hearts would be unable to increase their cardiac output during exercise sufficiently to cause a significant increase in work-load. However experiments with dogs do not support this argument. Thus, it has been shown that dogs with chronic cardiac denervation increase their cardiac output on exercise to the same extent as do control dogs (Donald and Shepherd 1964) and studies in racing greyhounds have shown that the capacity for maximal exercise is little reduced by cardiac denervation (Donald, Milburn and Shepherd, 1964). In greyhounds, the increase in cardiac output during the race could be blocked by propranolol in the dogs with cardiac denervation, and these dogs were unable to complete their race (Donald, Ferguson and Milburn, 1966) this indicates that the normal increase in cardiac output observed in dogs with cardiac denervation was achieved through circulating catecholamines, probably largely of adrenal medullary origin.

Thus we are left with the possibility that the cardiac sympathetic nerves release a substance(s) which is necessary for the induction of adaptive hypertrophy of the heart, or at least the hypertrophy evoked by chronic physical exercise and which might in fact in itself be the factor that induces the

biochemical events leading to hypertrophy of the cardiac muscle cell.

Since sympathetic nerves are known to release not only NA, but also secretory proteins, dopamine  $\beta$ -hydroxylase and chromogranins, as well (Smith *et al.* 1970 reviewed by Smith 1973) one cannot from this experiment alone form the hypothesis that NA is the essential link in inducing adaptive cardiac hypertrophy in trained rats. Thus there are three possibilities: either NA itself is sufficient, or the secretory proteins alone are required, or both NA and proteins are necessary for the induction of hypertrophy. The finding that chronic treatment of rats with low doses of isoprenaline gives rise to cardiac hypertrophy (references see above) supports the general hypothesis and is not inconsistent with the idea that secretory proteins, as well as NA itself, could be a factor inducing cardiac hypertrophy since the doses of isoprenaline used cause profound and prolonged drop in blood pressure which would result in reflex activation of the sympathetic nervous system and increased activity in cardiac sympathetic nerves (Stanton *et al.* 1969 Mueller and Axelrod, 1968 Mueller and Thoenen, 1971).

The finding that long term treatment of rabbits with either propranolol or practolol reduces the growth of the heart (Vaughan Williams and Raine 1974 Vaughan Williams *et al.* 1975) seems to indicate that the  $\beta$ -agonist action of NA is required for the growth of the heart in the sedentary animal but it should be remembered that propranolol administration decreases the amount of NA released in the heart on stimulation of the stellate ganglion (Playlor and Canon 1973) and thus probably also decreases the amount of secretory proteins released as well as blocking the  $\beta$ -receptors on the heart. Furthermore chronic propranolol treatment probably decreases preganglionic sympathetic nervous activity as well (Raine and Chubb 1977).

The present finding that the urinary excretion of NA is fairly well maintained in the guanethidine-treated rats and is increased during exercise, probably as a result of increased secretion of NA from the adrenal medulla indicates that the circulating levels of NA are not markedly different in sympathectomized and untreated rats. Thus the reason why cardiac hypertrophy is prevented by sympathectomy might be because the cardiac muscle cell only hypertrophies when it is exposed to the high local concentration of NA reached after release from the nerve endings, or if they are exposed to high local concentrations of the secretory proteins.

The fact that chemical sympathectomy did not abolish the cardiac hypertrophy caused by isoprenaline treatment indicates strongly that the trophic factor released from the sympathetic nerves is NA alone. After chemical sympathectomy high local synaptic concentrations of the secretory proteins would not occur this is because most of the serum dopamine  $\beta$ -hydroxylase activity found in normal rat serum is derived by release from sympathetic nerves, and little from the adrenals (Axelrod, 1972). Therefore by exclusion of the secretory proteins and on the evidence of the ability of isoprenaline to induce cardiac hypertrophy in the absence of cardiac sympathetic nerves we can conclude that NA must be the normal trophic factor. Although  $\alpha$ -agonists have been shown to exert effects on e.g. myocardial contractility (for references see Williams and Lefkowitz, 1978) it seems unlikely that isoprenaline would exert its trophic effect via  $\alpha$ -receptors as the ratio of  $\alpha$ -agonist activity to  $\beta$ -agonist activity is only 0.001-0.003 in this drug (Furchgott, 1972). Furthermore converting the  $\alpha$ -agonist activity of the isoprenaline doses given in the present study to an equivalent dose of NA one gets a NA dose in the region of 0.12-0.70  $\mu$ g per 24 h which is a very insignificant amount compared with the endogenous concentrations of NA in the circulation, not to mention the very much higher concentrations reached between the sympathetic nerve terminal and the effector cell. It therefore seems likely that isoprenaline exerts its trophic action via  $\beta$ -receptors. It appears highly likely that the trophic action of NA is also mediated via  $\beta$ -receptors.

It is noteworthy that the degree of cardiac hypertrophy obtained by isoprenaline treatment was actually greater in the sympathectomized rats (31% as compared to 24% in controls) and this observation, like the findings on salivary glands (see below) raises the possibility that denervation supersensitivity to the trophic actions of NA might occur.

Trophic actions of sympathetic nerves and/or catecholamines have been described in salivary glands, where the hypertrophy following repeated tooth amputation has been found to be mediated via the sympathetic nerves (Weils *et al.* 1961) and where various catecholamines induce hypertrophy and hyperplasia of the glands (see review by Hemme-Hagen 1972) and in chick embryo where NA has been shown to increase RNA synthesis (Calkers *et al.* 1970). Furthermore it has been suggested that  $\alpha$ -blockade with phenoxybenzamine interferes with early stages of liver regeneration in partially hepatectomized rats (Thrower *et al.*, 1973) and that catecholamines affect cell growth and cell division of *Tetrahymena pyriformis* possibly via  $\beta$ -receptors (Ivats *et al.*, 1969). It is therefore quite plausible that NA could exert trophic effects on the cardiac muscle cell and in this context the not uncommon occurrence of cardiac hypertrophy in patients suffering from pheochromocytoma deserves mentioning (Wessell and Craig 1968; Gerold and Jennings, 1972).

In summary therefore it appears likely that in exercise-induced cardiac hypertrophy it is NA released from cardiac sympathetic nerve endings and acting via  $\beta$ -receptors that is the link between the raised physiological demands and the biochemical events in the cardiac muscle cell leading to compensatory hypertrophy and that increased cardiac work load in itself does not lead to adaptive hypertrophy.

## CONCLUSIONS to Chapter 6

(1) Chemical sympathectomy produced by chronic guanethidine-treatment is associated with reduction in weight gain and with the higher dose regime reduction in actual body growth. There is also reduction in the urinary excretion of NA, the extent of which is likely to be determined by the denervation supersensitivity of the vasculature. Circulating NA is probably largely derived from compensatory selective increase in the secretion of NA from the adrenal medulla. The weight of the adrenal glands in guanethidine-treated rats was increased perhaps indicating that there is increased adrenocortical activity as well.

(2) The chemically sympathectomized rats easily tolerated the exercise programme and the regular physical activity further accentuated the adaptive increase in adrenal NA content seen in sedentary sympathectomized rats. However although the training programme was identical to that in untreated rats, the sympathectomized rats did not show the compensatory cardiac hypertrophy found in the untreated rats. It is argued that this indicates that the adaptive cardiac hypertrophy produced by chronic exercise is not caused by direct effect of the increased work load on the cardiac cell but is instead mediated by release of trophic factor from cardiac sympathetic nerves.

(3) The finding that the generalized cardiac hypertrophy caused by isoprenaline treatment was not abolished by chemical sympathectomy suggests that the trophic factor released from cardiac sympathetic nerves at chronic exercise is NA, rather than any of the secretory vesicle proteins, and that NA exerts its trophic effect on the cardiac muscle cell via  $\beta$ -receptor.

## SOME WIDER IMPLICATIONS OF THE RESULTS OF THESE STUDIES

### *The role of the sympathetic nervous system in the bradycardia of the athlete's heart*

It has been a commonly held belief that the relative bradycardia found in athletes and in chronically exercised animals is due to an excessive vagal tone although the experimental evidence for this is both indirect and unconvincing (see Chapter 1). The finding, confirmed by the use of three different methods (Chapters 3 and 5), that the turnover of NA in cardiac sympathetic nerves is significantly lower in trained rats than in controls, both during rest and during exercise, provides an alternative explanation for the bradycardia of the athlete's heart. Since the sensitivity of the isolated heart to the chronotropic actions of NA is not altered in trained rats (Section G, Chapter 3) it would appear that the bradycardia of the trained rat could be accounted for by this decrease in nervous activity of cardiac sympathetic nerves. That a similar situation is found in man is suggested by the observation of Bjurstedt *et al.* (1974) that in well-trained human individuals the resting heart rate is not slowed by  $\beta$ -blockade with propranolol.

Further independent evidence supporting the concept of decreased nervous activity in cardiac sympathetic nerves in trained rats is provided by the work of Lin and Horwath (1972). These authors concluded from their experiments in rats treated with propranolol and/or atropine that there is a decrease in both sympathetic and parasympathetic tone to the heart of trained animals, but that the decrease in sympathetic tone is the more prominent.

Eklöf *et al.* (1973) found that chemical sympathectomy by 6-OHDA diminished the difference in heart rate during exercise normally seen between trained rats and controls, which supports the concept of lower cardiac sympathetic tone in trained rats. As the difference in heart rate during exercise was not entirely abolished by 6-OHDA treatment the authors concluded that some other factor must be contributing to the observed relative bradycardia. However, since their 6-OHDA treated rats showed evidence of cardiac denervation supersensitivity to circulating catecholamines, the remaining difference in rate after sympathectomy could be entirely explained by the fact that chronically exercised rats, whether trained by swimming (see Section D) or forced running (N.O. Sjöstrand *et al.* unpublished observation) have smaller rise in circulating catecholamine levels during exercise than untrained rats do.

As shown in Section G, the intrinsic rate of intact isolated hearts from chronically exercised rats is the same as that of controls, and therefore a decreased intrinsic heart rate is very unlikely to account for the relative bradycardia of the trained rat.

A finding of decreased sympathetic tone to the heart does not of course exclude the possibility that simultaneous increases in vagal tone could be present. However as atropine causes smaller rise in heart rate in trained rats than in controls even if the rats have been given propranolol as well (Tipton and Taylor 1965; Lin and Horwath 1972) there seems to be little experimental support for such a concept. Likewise human athletes show smaller heart rate increases after atropine than controls do (Henschen 1921; Keul 1926).

In summary therefore it appears that the bradycardia seen in trained rats is entirely accounted for by the decrease in cardiac sympathetic nervous activity and it seems likely that the same is true in human athletes as well.





at normotensive levels, and in this respect being if anything more effective than  $\alpha$ -methylglutamate, it failed to prevent the occurrence of cardiac hypertrophy both in rats treated when hypertension was established and in rats started on treatment prophylactically before hypertension had occurred. The authors interpret their findings as suggesting that the renin-angiotensin system might play a permissive role in enhancing cardiac hypertrophy (Sen *et al.*, 1974). Obviously renin doesn't qualify as a candidate for "a final common pathway" in inducing cardiac hypertrophy as compensatory cardiac hypertrophy occurs in "low renin" types of hypertension (see Sen *et al.* 1974 for refs.) However the results of Sen *et al.* (1974) fit well with the hypothesis under discussion. Thus  $\alpha$ -methylglutamate has a central hypotensive action leading to a relative bradycardia as well as a decrease in lumbar sympathetic impulse outflow (Ingenito *et al.* 1970; Baum *et al.* 1972). It would therefore tend to diminish the exaggerated neurally mediated cardiovascular responses precipitated by external stimuli in spontaneously hypertensive rats. The hypotensive action of hydralazine on the other hand is predominantly due to relaxation of vascular smooth muscle and both heart rate and stroke volume are increased, presumably due to reflex-induced increases in cardiac sympathetic nervous activity as hydralazine-induced tachycardia can be prevented by ganglion blockers and  $\beta$ -adrenergic blocking agents (see Nickerson and Ruedy 1975). Hydralazine would not, therefore diminish pathologically increased activity in cardiac sympathetic nerves in spontaneously hypertensive rats and so according to the hypothesis, would not inhibit the induction of cardiac hypertrophy. Similarly Messon *et al.* (1968) noted dissociation between the blood pressure control by hydralazine in experimental renovascular hypertension and the persistence of cardiac hypertrophy and this might again be interpreted as supportive evidence for a role of cardiac sympathetic nerves in inducing compensatory cardiac hypertrophy.

A further piece of evidence supporting the hypothesis is the finding of Fernandes *et al.* (1976) that propranolol treatment reduced the development of cardiac hypertrophy in renal hypertensive rats even though their blood pressure remained as elevated as in the untreated renal hypertensive rats.

In a recent paper pertinent to the hypothesis under discussion Cohen (1974) has suggested that in fact myocardial adrenergic nerve terminals are not required for the cardiac hypertrophy induced by desoxycorticosterone-induced hypertension on the basis of experiments with rats treated with 6-OHDA. However there are several aspects of Cohen's experiments that can be criticized. First, the dose of 6-OHDA used (20 mg/kg, twice daily on two successive days weekly i.p.) is not sufficient to produce maximal chemical sympathectomy (see Thoenen 1972). Second, no allowance was made for the likely regeneration of sympathetic fibres which occurs rapidly in adult animals. Even after much larger doses of 6-OHDA than those used by Cohen (1974), regenerating terminals are seen in the myocardium after 3-8 days (Lonsz *et al.* 1975) and after relatively small doses the regeneration is even more rapid (Maimon and Sachs, 1968; Goldman and Jacobowitz, 1971). Third, the degree of sympathectomy was only assessed qualitatively by fluorescence histochemistry and the results presented show considerable residual catecholamine-fluorescence in the heart of treated animals. Thus, Cohen's (1974) results are not sufficiently critical test of the hypothesis that the cardiac sympathetic nerve is the final common path in many etiologies of cardiac hypertrophy.

It can therefore be concluded that there is considerable body of evidence that cardiac sympathetic nervous activity is increased in all the pathological conditions causing adaptive cardiac hypertrophy discussed above and that there are some further indications that this increased nervous activity may initiate the adaptive hypertrophy.

**What is then the role of other hormones in cardiac hypertrophy?** Patients suffering from pituitary tumours producing growth hormone are frequently noted to develop cardiac hypertrophy (see review by Cohen 1974) and hypophysectomy causes a relative reduction of cardiac size which is only fully reversed if substitution therapy of both growth hormone and thyroxine is given (see review by Wilmshorn 1971). Neither the presence of growth hormone nor that of thyroxine is however necessary



essions the body weight remained the same as that of sedentary rats. As far as adaptive changes of the sympatho-adrenal system is concerned the intermittently trained rats showed values of noradrenaline content in heart and adrenal medulla that were intermediate between those of sedentary controls and those of rats subjected to daily exercise and, furthermore the excretion of noradrenaline and adrenaline in the urine following an exercise session was less than that of untrained rats but greater than that of the daily-trained group.

B. Comparing physically trained rats with cold-acclimated rats the following main points emerged:

- (a) Both groups developed the same degree of cardiac and adrenal hypertrophy but only cold-acclimated rats showed enlargement of the submandibular salivary glands.
- (b) Cardiac noradrenaline content increased in both the trained and cold-acclimated rats, but more so in the former group which was the only group to show an increase in the concentration of noradrenaline in the heart. The noradrenaline content in the spleen was raised in the trained rats but decreased in the cold-acclimated group whereas only the cold-acclimated rats showed an increase in the noradrenaline content of the submandibular salivary glands. The content of noradrenaline in the adrenal medulla increased to the same extent in both the experimental groups, but only the trained group showed an increase in the adrenaline content of the adrenal medulla. The noradrenaline content in the brain of cold-acclimated rats was unaltered in contrast to the increased values found in trained rats. It is suggested that these differences in adaptive responses reflect differences in the degree of activation of the various parts of the sympatho-adrenal system caused by the two experimental conditions studied.
- (c) Trained as well as cold-acclimated rats excreted less noradrenaline in the urine during rest than controls did. Following a session of swim-exercise only the trained not the cold-acclimated group displayed smaller increment in urinary noradrenaline excretion than that seen in sedentary warm-acclimated rats. After cold stress however smaller rise in the amount of noradrenaline excreted in the urine was found in both the trained and the cold-acclimated rats than in the control group.

D. Inhibition of noradrenaline synthesis following treatment with  $\alpha$ -methyltyrosine leads to alterations in turnover rate about 4 hours after the treatment begins slowing down the turnover of noradrenaline in the brain and an acceleration occurs in the heart, during the 4 to 18 h period. Furthermore at the same time as the turnover in the brain slows down, the pre-existing difference in cardiac noradrenaline turnover between trained and untrained rats disappears. Thus, the inhibition of noradrenaline synthesis by  $\alpha$ -methyltyrosine is a completely unsuitable method for studying the normal physiological activity of noradrenergic neurons, both central and peripheral, at any time later than 4 hours after administration of the drug.

7. In order to study the role of sympathetic nerves in adaptive cardiac hypertrophy rats were subjected to permanent chemical sympathectomy by daily injections of high doses of guanethidine (30–60 mg/kg BW) for six weeks.

- (a) This treatment led to an extensive destruction of sympathetic ganglion cells with a decrease of organ noradrenaline content of 90% in the spleen and the submandibular glands and of 83% in the heart there was compensatory 45% increase in adrenal noradrenaline content. Guanethidine-treated rats at rest excreted 45% less noradrenaline in the urine than the untreated rats, but the resting noradrenaline excretion was by no means maximal since, after exercise the guanethidine-treated rats increased their excretion of noradrenaline by exactly

the same proportion as the untreated rats did. It is suggested that the low level of noradrenaline excretion by resting guanethidine-treated rats, in spite of their ability to excrete more, is reflection of the degree of denervation supersensitivity that develops in the vascular smooth muscle.

- (b) The guanethidine treatment caused reduction in weight gain and in the higher dose also an actual impairment of body growth as expressed by final body length.
- (c) Chemical sympathectomy with guanethidine treatment completely abolished the adaptive cardiac hypertrophy in rats subjected to daily exercise but did not abolish the cardiac hypertrophy induced by isoprenaline treatment.

## MAIN CONCLUSIONS

The questions posed at the end of Chapter 1 can now be answered.

1 Daily physical exercise induces adaptive changes in both central and peripheral noradrenergic neurons in the rat. The stores of noradrenaline are increased in brain, heart and adrenal gland, and at least transiently in the spleen as well. There are changes in (nervous) activity in both central and peripheral noradrenergic neurons, but whereas the activity is increased in the brain, the activity of peripheral sympathetic neurons is decreased in both heart and spleen and probably in the vasculature as well. It is suggested that the decrease in activity in peripheral sympathetic neurons is secondary to an increase in inhibitory activity by central noradrenergic neurons. This central effect may in turn be at least partially a reflex adjustment to the increased sensitivity to the vasoconstrictor actions of noradrenaline that occurred in the trained rats.

2 The bradycardia of the "athlete's heart" may be largely or entirely due to the decrease in cardiac sympathetic nervous activity caused by training.

3 Physical training appears to induce "cross tolerance" to cold stress, while cold-acclimation does not lead to "cross tolerance" to acute exercise.

4 The adaptive cardiac hypertrophy produced by chronic exercise is not caused by a direct effect of the increased workload on the cardiac muscle cell, but is instead mediated by release of noradrenaline from cardiac sympathetic nerves. Furthermore, increased activity of cardiac sympathetic nerves may be the final common pathway in all forms of compensatory cardiac hypertrophy.

- CARLSSON A and S WALDECK A fluorimetric method for the determination of dopamine (3-hydroxytyramine) Acta physiol scand 1970 44 293-298
- CARLSSON M and J DENCKER C GRIMBY and J NORDENFALD Circulatory studies during physical exercise in mentally disordered patients II Effects of physical training in patients with and without clinical treatment of chlorpromazine Acta med scand 1968 184 511-516
- CARLSSON L.A. B PERROW and S ZETTERQUIST Studies on peripheral circulation and metabolism in man III Oxygen utilization and lactate-pyruvate formation in the legs at rest and during exercise in subjects with hyperkinetic circulation and low physical working capacity (Vasoregulatory Anemia) and in healthy female Acta med scand 1962 177 399-400
- CARPI A and A OLIVIERO Urinary excretion of catecholamine in the immunosympathectomized rat: balance phenomena between the adrenergic and noradrenergic systems J. Neuropharmacol 1964 3 427-431
- CARR L.A. and K.E. MOORE Effects of meprobamate and  $\alpha$ -methyltyrosine on brain catecholamines and the pituitary-adrenal response in stress Neuroendocrinology 1968 3 285-302.
- CARRIER O J Role of calcium in postjunctional super sensitivity Fed. Proc 1975 34 1975-1980
- CHAMPPLAIN J de Degeneration and regrowth of denervated nerve fibres in the rat peripheral tissues after 6-hydroxydopamine Can J Physiol Pharmacol 1971 49 345-355
- CHAMPPLAIN J C and M.R. van AMERINGEN Role of sympathetic fibres and of adrenal medulla in the main source of catecholamine homeostasis: a comparative and hypertensive rats In Frontiers in Catecholamine Research 1973 pp 879-884. Eds E. Usdi and S. Snyder Pergamon Press New York Toronto Oxford Sydney New Delhi
- CHAMPPLAIN J de P GAUTHIER G FORLIER M.R. van AMERINGEN and R.A. MADRAC Compensatory mechanisms following peripheral chemical sympathectomy I Chemical Tools in Catecholamine Research 1973 I pp 231-238 Ed G Jonsson T Malmfors and Ch. Seth North-Holland Publishing Company Amsterdam
- CHAMPPLAIN J de R.A. MEYLER and J AXELROD Turnover and synthesis of norepinephrine in experimental hypertension in rat Circ. Res 1966 29 285-291
- CHANG C.C. A sensitive method for spectrofluorimetric assay of catecholamines Int. J. Neuropharmacol 1964 3 643-649
- CLARK D.W.J. Effects of immunosympathectomy on development of high blood pressure in genetically hypotensive rat Circ. Res 1971 28 330-336
- COHEN J Role of endocrine factors in the pathogenesis of cardiac hypertrophy Circ. Res 1974 35 Suppl. II 49-57
- COHEN J J.M. ARONSTY and M.G. ROSENFIELD Derivatives of thyroxine-induced hypertrophy in rat Circ. Res 1968 18 388-397
- COOPER T Surgical sympathectomy and adrenal function Pharmacological Reviews 1966 18 611-618
- COOPER T J.W. GILBERT J R.D. BLOODWELL and J.R. CROFT Chronic experimental cardiac denervation by regional neurolytic blockade: implications for the operation verification of the denervation and its effect on myocardial catecholamine Circ. Res 1961 9 275-281
- COOPER T V.L. WILLIAMS M. JELLINEK and C.R. WARLEN Heart denervation: effects on myocardial catecholamines and histamine Circulation 1962 128 40-41
- CORRODI R. K. FUXE and T. HÖNFELT A possible role played by central noradrenergic neurons in thermoregulation Acta physiol scand 1967 71 224-232
- CORRODI R. K. FUXE and T. HÖNFELT The effect of neuroleptics on the activity in central catecholamine neurons Lif. Sci 1967 6 767-774
- CORRODI R. K. FUXE and T. HÖNFELT The effect of immobilization on the activity of central noradrenergic neurons Lif. Sci 1968 7 107-112
- COTRACCI R. and L.C.F. BAXSON Central effects of an inhibitor of tyrosine hydroxylase Psychopharmacologia (Berl.) 1966 10 116-123
- COSTA E and J.L. MEYER Regulation of biosynthesis of catecholamines and serotonin in the CNS Ann. Rev. Pharmacol 1974 14 491-511
- COUTLER V.B. and L.D. CARLSON Regulation of heat production in cold-adapted Exp. Biol. (N.Y.) 1956 22 845-849
- COX R. and B. POTTERMAN The effect of ambient temperature on the release of noradrenaline, dopamine, 3-hydroxytyramine and acetylcholine from brain Br. J. Pharmacol 1967 31 336-346
- CRILEY J and K.E. ALDRIDGE Effect of brain lesion on myocardial function Am. Rev. Physiol. 1967 24 536-542

- ELIAS E.I. and J. FRANKLIGER Brain waves and emotional trees J. Psychia 1966 4  
189-196
- BLOOM C.M. and A.B. LEON Is erection f age and exercise on the heart and its blood supply  
Lab. Invest. 1970 22 160-165
- ELSTER T.H., R.C. HALL and I.E. HUGHES An assessment f the coeliac ganglion sympathectomy  
induced by guanethidine J. Pharm. Pharmacol. 1974 26 53-57
- ROCK A.V., C. WARCANTREY, D.B. WILL & P. FELLING and L.M. HONKHAL Studies in muscular  
activity III Dynamical changes occurring in man work. J. Physiol London 1928  
64 136-161
- WILLINGER Über die idiopath. Myohypertrophie und die Dilatation des Herzens Arch. S.d.  
pathol. Ana. Marbury 1886 p 30
- BOULE F., K. FUNK and F. LUDWIG On the function f central catecholamine neuron the  
role in cardiovascular and renal mechanisms Ann. Chim. (Chem. Path. Pharmacol.)  
1972 4 657-697
- WELTER, C.F., R.L. HODGSON and J.B. CHUTE Intrinsical re and cholinergic sensitivity f  
isolated tris from trained and sedentary re Proc. Soc. Exp. Biol. Med. 1973 144  
364-367
- BORCHARD F. The adrenergic nerves f the normal and hypertrophied heart. Normal and  
Pathological Anatomy 1978 33 pp 1-46 Eds V. Bergmann and W. Dörer  
Georg Thieme Publishers Stuttgart
- WHALEY J. A. WELLY A.M. LALLIBANT and A.M. WEALEY Temps de aneuvellenn de la  
sécrétion tissulaire influence de traitement po l'adétylparatyroline sur la  
pénétation de dispersion de la noradrénaline endogène 1 finction de la noradrénaline  
dans différents organes de re Physiologie Arch. 1971 329 341-351
- WHEELER S.B., R. COSTA, A. DIAMAC, B.H. KEFF and R.E. WOODLICK Application f steady state  
kinetics to the estimation f synthesis rate and turnover time f tissue catecholamines  
J. Pharm. Exp. Therap. 1966 154 493-498
- WRIGHT M.J. Coeliac ganglion responses f flowing immunological sympathectomy Circ. Res.  
1964 15 141-147
- WRIGHT L., T.R. CONRADSON, B. OLSON and S. VAMMAKIDIS High trial monophasic action  
po central and effective refractory period in alction physical training and central  
heart re Cardiovasc. Res. 1976 10 160-168
- WRIGHT A.L. J. Morphology f the re f cardiac hypertrophy in Cardiac Hypertrophy 1971  
pp 11-18 Ed. W.R. Alpert Academic Press Inc. New York and London
- WRIGHT G. and M. COSTA Growth and degeneration f adrenergic nerves I Adrenoleukia  
Reviews 1975 pp 107-128 Chapman and Hall Ltd London
- WRIGHT G., R. KYLES, B.J. GARDNER, J. HEATH and JAMES A new method f destroying  
adrenergic nerves in adult animals using guanethidine Br. J. Pharmacol. 1971 43  
295-301
- WRIGHT A.B.C. Acclimatisation re wild in the Antarctic Nature (Lond.) 1949 162  
132-133
- WRIGHT, R.J. and G.B. THOMP Effect f chronic exercise on transcoelomic function and  
structure in the re J. Appl. Physiol. 1971 21 680-693
- WRIGHT P.M., C.R.J. EUSTICE and M. SANDLER On catecholamine and 3-hydroxyindole metabolism  
in immunosympathectomized rats Br. J. Pharmacol. 1966 24 70-76
- WRIGHT F.R., A.A. FAIRMAN and G.J. HODGSON Central neural regulation f heart and blood  
vessel in man Physiol. in Man 1973 2 1-33
- WRIGHT C.M., F.F. GIORGI and A. CASTI The effect f noradrenaline on polyoma and RNA  
synthesis in the chick embryo J. Endocrinol. 1970 44 113-116
- WRIGHT E.A. and J.J. PARK, Kallergren f the guinea pig salivary gland caused by catechol  
amine or tooth extraction. Europ. J. Pharmacol. 1968 2 371-376
- WRIGHT The Wisdom f the Body 1932 W. W. Norton Company New York.
- WRIGHT B., J.R. LINTON and R.R. LINTON The effects f neural metabolites on  
sec on Am. J. Physiol. 1971 21 153-162
- WRIGHT S., K.F. WILSON, E.M. BRIGHT, V. MERRIN and R.M. MOORE Some  
physiologic f animals surviving complete enucleation f sympathetic III  
Am. J. Physiol. 1929 89 94-107
- WRIGHT B., S. QUTALDO S. BRITTON and E.M. BRIGHT Studies on the  
f endocrine glands XII The secretion in the  
body excretors Am. J. Physiol. 1971 21 464-506
- CARLSON and LINDQVIST 1  
metatyrosine Ac. physiol. 1  
1971 21 67-94

## REFERENCES

- ADAMS T and E J REIBELING Human physiological response to standardized cold stress modified by physical fitness J. Appl. Physiol 1958 11 226-230
- ALLOTTI A. and R. WEINER, The regulation of noradrenaline synthesis and sympathetic nerve effect of nerve stimulation cocaine and tetraethylammonium releasing agent Proc. Natl Acad Sci. USA 1966 36 1491-1496
- ALPERT N.R. Cardiac Hypertrophy 1971 pp 1-641 Academic Press Inc New York and London
- ANDERSON N.E., R. COHEN, E. FURE, R. WICKFELT, T. WICKFELT, R. HYDIN and T. SVENSSON Evidence for central noradrenaline receptor stimulation by lisdoline Life Sci 1970 9 513-523
- ANGELIETTI F.V. and R. LEVI-MONTALCINI Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine Proc. Natl Acad Sci USA 1970 65 114-121
- ANGELIETTI F.V. and R. LEVI-MONTALCINI Growth inhibition of sympathetic cells by some adrenergic blocking agents Proc. Natl Acad Sci USA 1972 69 84-88
- ASTRAND P.-O., Z.E. CROFT, B. SALTIN and J. STEENBERG Cardiac output during submaximal and maximal work J. Appl. Physiol 1964 19 268-274
- ASTRAND P.-O. and K. RODAHL Textbook of Work Physiology 1970 McGraw Hill New York
- AXELROD J. Dopamine  $\beta$ -hydroxylase Regulation of its synthesis and release from nerve terminals Pharmacol. Rev 1972 24 233-243
- BARTASOVA D., M. CERNY, B. KOSCHNY, O. POUHA, K. KAKOSAN, Z. TURK and M. VIZEK, The growth of the muscular cell genome parallel to the rate of various forms of cardiomyopathy J. Physiol (London) 1969 200 285-295
- BAIN T. A.T. SCHOPPER and L.L. WASSER, Conduction of the central nervous system to the action of several antihypertensive agents (methyldopa, hydralazine and guanethidine) J. Pharmacol. exp. Therap 1972 183 135-143
- BERENSON B.A., S. SPECTOR and J.M. TOWER Resistance of noradrenaline to blood vessel constriction by 6-hydroxydopamine or immunosympathectomy Br. J. Pharmacol 1972 44 10-16
- BICKLER R. R., K. WILLIAMS and E. WOLFESEN Some observations on the synthesis and storage of catecholamines in the adrenal medulla cell of the suprarenal medulla Acta Physiol Scand 1960 50 124-131
- BEVILACQUA S. Studies on the regulation of the circulation in man. With special reference to the role of volume and the effect of muscular work, body position and artificially induced variations of heart rate Acta Physiol Scand 1962 27 suppl 200 1-34
- BEVILACQUA S. A. WOLFESEN and S. JOHNSON Circulatory modification in well trained athletes and during heavy exercise with special reference to stroke volume and its influence on body position Acta Physiol Scand 1963 27 28-50
- BEVILACQUA S. S. and J.T. SHEPHERD Regulation of the circulation during exercise in man. Physiol. Rev 1967 47 178-213
- BEZMAK H. The behaviour of the weight of the heart and the blood pressure of the rats under different conditions J. Physiol (London) 1954 124 44-63
- BEZMAK H., B. KOSCHNY and G. THOMAS Regulation of renal hypertrophies of various origin Can. J. Pharmacol 1969 47 579-586
- BEZMAK A. von and J. PERLES Über den Zusammenhang d. Nierenarteriendruckhypertrophie i. d. Körperlichen Arbeit und mit dem Gehalt der Nahrung an Vitamin B. Pflügers Arch. f. Physiol 1935 224 181-189
- BEZMAK A. von and L. SARKADY Über die Wirkung der Arbeit auf das Wachstum und Gewicht der Nierengänge Pflügers Arch. f. Physiol 1934 224 157-163
- BRAGAT S. The influence of sympathetic nervous activity on cardiac catecholamine level J. Pharmacol. exp. Therap 1967 157 74-80
- BRAGAT S. and E. FRIEDMAN Effect of valved main source of cardiac catecholamine concentration: importance of synthesis and re-uptake Br. J. Pharmacol 1969 27 24-31
- BICKLOW L.S., B. BAINMAN, E. WEIL-MALHERBE and S. UDENFRIEND Inhibition of synthesis of catecholamine and their metabolites following the administration of phenoxybenzamine Br. J. Pharmacol 1969 2 363-371
- BURSTENTON N. G., R. WOLFESEN and G. TYDEN Action of and effect of propylthiouracil on diastolic pressure Acta Physiol Scand 1974 99 491-500
- ELIAS L.L. and J. ALLISON Response of noradrenergic amines to reuptake and degradation J. Pharmacol. exp. Ther 1969 169 258-263
- ELIAS L.L. and J. ALLISON Relationship of reuptake and degradation to dopamine and homovanillic acid Life Sci 1971 10 11-1149

- ELIAS K.L. and J. ZWANEKKE. Brain stress and emotional stress J. Psychiat. Res. 1964 4 189-193
- ELSON, H.M. and A.S. LEON. Interaction of age and exercise on the heart and its blood supply Lab. Invest. 1970 22 160-165
- ELTZER T.R., R.C. KALL and I.K. HUGHES. An assessment of the cardiovascular sympathetic activity induced by guanethidine J. Pharm. Pharmacol. 1976 28 33-37
- ROCK, A.V., C. WISNIAKOWSKI, H.B. DILL, A. POLLING and L.M. HINDRICH. Studies in muscular activity III. Dynamical changes occurring in man work. J. Physiol. London 1928 64 136-161
- KOLLIKER Über die idiopath. Parasympathotrophie und die Dilatation des Herzens Arch. d. d. pathol. Anat. Histol. 1886 p. 30
- BOULE P., K. FUXE and P. LIDENHEIM. On the function of some catecholamine neurons: their role in cardiovascular and renal mechanisms Rev. Comp. Chem. Path. Pharmacol. 1972 4 657-697
- MEYER C.P., R.L. HUGHESON and J.R. CHUTE. Intrinsic and cholinergic sensitivity of isolated trachea from trained and sedentary rats Phys. Soc. Exp. Biol. 1973 164 344-347
- BOURDARD P. The adrenergic nerves of the normal and hypertrophied heart. Normal and Pathological Anatomy 1978 32 pp. 1-68 Eds V. Bergmann and W. Doerr Georg Thieme Publishers Stuttgart
- BRADY J., A. KELLY, A.M. LALLEMENT and A.M. BRADY. Temps de recouvrement de la noradrénaline tissulaire influencé du traitement par le  $\alpha$ -méthylparasympatholique sur la vitesse de disparition de la noradrénaline endogène. La fixation de la noradrénaline dans différents organes de rat PTI/gene Arab. 1971 329 341-351
- BRIDGES R.B., L. COSTA, A. DIABAC, H.R. MEYER and H.L. SPICKER. Application of steady state kinetic to the estimation of synthesis rate and turnover time of catecholamines J. Pharmac. Exp. Therap. 1968 124 493-498
- BRIDGES R.B. Cardiac color responses following immunological sympathectomy Circ. Res. 1964 15 161-167
- BRONSON L., T.R. COMMISSIONER, OLSON and R. VANMANDEL. Right atrial monophasic action potential and effective refractory periods in relation to physical training and maximal heart rate Cardiology, Res. 1976 10 160-168
- BROWN A.L. J. Morphological factors in cardiac hypertrophy J. Cardiac Hypertrophy 1971 pp. 11-18 Ed. H.R. Alpert Academic Press Inc. New York and London
- WHITLOCK R. and K. COSTA. Growth and degeneration of adrenergic neurons. In Adrenergic Neurons 1975 pp. 107-128 Chapman and Hall Ltd London
- KUMAROCK G., S. KALAS, S.J. GARRON, J.W. HEATH and V. JAMES. A new method of destroying adrenergic nerves in adult animal using guanethidine Br. J. Pharmacol. 1971 42 295-301
- BUTLER A.R.C. Acclimatization to cold in the Antarctic Science (Lond.) 1949 163 132-133
- BUCK R.J. and G.D. THAYER. Effect of bronchial exercise on endocortical function and structure in the rat J. Appl. Phys. 1971 21 840-843
- CHEN, Y.H., C.R.J. KUTYER and H. SANDLER. Catecholamine and 3-hydroxyindole metabolism in immunosympathectomized rat Br. J. Pharmacol. 1969 24 70-78
- CHALANCO Y.H., A.A. FAIRER and G.J. HUGHESON. Central adrenal regulation of heart and blood vessels in man Progress in Neurobiol. 1975 2 1-33
- CHILKOTSKA C.M., P. GIORGI and A. CASTI. The effect of noradrenaline on polyamine and RNA synthesis in the chick embryo J. Endocrinol. 1970 44 115-116
- CHOUFOS S.A. and J.J. PARK. Halargenone of the guinea pig salivary gland secreted by an ethyl anise touch operation Europ. J. Pharmacol. 1968 2 371-378
- CLARK, S. The Mind of the Body 1932 W. Norton Company New York.
- CARSON S., J.R. LINTON and B.R. LINTON. The effect of animal metabolism on adrenal secretion Am. J. Physiol. 1934 21 153-162
- CARSON S., R.F. NEWTON, E.M. BRIGGS, MERRIN and R.M. HODGE. Some aspects of the physiology of animals surviving complete unilateral sympathetic denervation of the sympathetic nervous system Am. J. Physiol. 1929 87 94-107
- CARSON W. and O. QUINTELL. Studies on the conditions of activity of endocrine glands. XII. The role of adrenal medulla in the hormonal control of body temperature Am. J. Physiol. 1927 78 464-506
- CARLSON A. and K. LINDQVIST. In vivo deacetylation of  $\alpha$ -methyl DOPA and  $\alpha$ -methyl metatyrosine Acta Physiol. Scand. 1974 87 94



- CARLSSON A and E WALDECK A fluorimetric method for the determination of dopamine (3-hydroxytyramine) *Acta physiol scand* 1958 44 293-298
- CARLSSON E and J HENCKELS, G GRIMBY and J HOGSTEDAL Circulatory studies during physical exertion in mentally disordered patients II Effects of physical training on facts with and without administration of chlorpromazine *Acta med. scand* 1968 184 511-516
- CARLSSON L.A., B PERSSON and S ZETTERQUIST Studies on peripheral circulation and metabolism in man III Oxygen utilization and l-tyrosine formation in the legs at rest and during exercise in subjects with hyperkinetic circulation and low physical working capacity (Vasoregulatory Asthenia) and in healthy females *Acta med. scand* 1962 172 399-400
- CARPI A and A OLIVIERO Urinary excretion of catecholamine in the immunosympathectomized rat: balance phenomena between the adrenergic and noradrenergic system *J. Neuropharmacol* 1964 3 427-431
- CARR L.A. and E.K. MOORE Effect of reserpine and -methyltyrosine on brain catecholamines and the pituitary-adrenal response to stress *Neuroendocrinology* 1968 3 385-392.
- CARRIER, O. J. Role of calcium in postjunctional opernsensitivity *Fed. Proc* 1975 34 1975-1980
- CHAMPLAIN J. de Degeneration and regrowth of denervated nerve fibres in the peripheral tissues of 6-hydroxydopamine *Can J Physiol Pharmacol* 1971 49 345-355
- CHAMPLAIN J. de and M-H van AMERIGHEN Role of sympathetic fibres and of adrenal medulla in the maintenance of cardiovascular homeostasis in normotensive and hypertensive rats *In: Frontiers in Catecholamine Research* 1973 pp 839-864. Ed. K. Ueda and S. Snyder. Pergamon Press, New York Toronto Oxford Sydney Braunschweig
- CHAMPLAIN J. de, P. GAUTHER, G. FOULIER, M-H. van AMERIGHEN and R.A. MADEAU Compensatory mechanisms following peripheral chemical sympathectomy *In: Chemical Tools in Catecholamine Research* 1975 1 pp 231-238. Ed. G. Jonsson, T. Melander and Ch. Sacchi. North Holland Publishing Company, Amsterdam
- CHAMPLAIN J. de, R.A. MULLER and J. AXELROD Turnover and synthesis of norepinephrine in experimental hypertension in rats *Circ. Res* 1969 25 285-291
- CHANG C.C. A sensitive method for spectrophotofluorimetric assay of catecholamines *In: J. Neuropharmacol* 1964 3 643-649
- CLARK D.W.J. Effects of immunosympathectomy on development of high blood pressure in genetically hypertensive rats *Circ. Res* 1971 28 330-336
- CORNE, J. Role of endoneurial sheath in the pathogenesis of cardiac hypertrophy *Circ. Res* 1974 35 Suppl II 49-57
- CORNE, J., J.M. ARNETT and M.O. ROSENFELD, Derivatives of thyroxine-induced hypertrophy in rat *Circ. Res* 1966 18 308-317
- COOPER T. Surgical sympathectomy and adrenal function *Pharmacol. Reviews* 1966 18 611-618
- COOPER T., J.V. GILBERT, J. R.D. BLOODWELL and J.R. CHOUT Chronotropic cardiac denervation by regional neurolysis. Description of the operation, verification of the denervation and its effect on myocardial catecholamine *Circ. Res* 1961 9 275-281
- COOPER T., V.L. WILLIAMS, H. JELLINGER and C.R. SHARON Neurotransplantation: Effect on myocardial catecholamines and histamine *Science* 1962 126 40-41
- CORRODI H., K. FUXE and T. HÖRTEL The role played by central monoamine neurones in thermoregulation *Acta physiol. scand* 1967 71 224-232
- CORRODI H., K. FUXE and T. HÖRTEL The effect of neuroleptics on the activity in central catecholamine neurones *Life Sci* 1967 6 767-774
- CORRODI H., K. FUXE and T. HÖRTEL The effect of immobilization stress on the activity of central monoamine neurones *Life Sci* 1968 7 107-112
- CORRODI H. and L.C.F. HANSON Central effect of an inhibitor of tyrosine hydroxylase *Psychopharmacologia (Berl)* 1966 10 116-125
- COSTA E and J.L. MEIN Regulation of biosynthesis of catecholamine and release of the CNS *Ann Rev Pharmacol* 1974 1 491-511
- COTTELL W.H. and L.D. CARLSON Regulation of the production of 14-adapted *Proc. Soc. exp. Biol. (N.Y.)* 1956 73 845-849
- COX B. and D. POTKINIAK The effect of ambient temperature on the release of norepinephrine and acetylcholine from brain *Br. J. Pharmacol* 1967 21 356-364
- CRONE J. and E.K. ALDRIDGE Effect of heart exercise on myocardial function *Am. Heart J* 1967 7 334-342

- DARLSTROM A. The intraneuronal distribution of noradrenaline and the transport and life-span of amine storage granules in the sympathetic adrenergic neuron. M.D. Thesis Ethna. 1966
- DARLSTROM A. and K. FUXE. Evidence for the existence of monoamine neurons in the central nervous system. II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems. Acta physiol. scand. 1965 94 Suppl. 247 7-36
- DARLSTROM A., K. FUXE, M. NYA TU and B.R. KILTERSTROM. Observations on adrenergic innervation of dog heart. Am. J. Physiol. 1965 209 689-692
- DEMECIER L. The influence of physical effort on the heart. Acta med. scand. 1970 53 738-743
- DE CHANT W.C. and R.W. KYALL. An excitatory action of 5-hydroxytryptamine on sympathetic preganglionic neurones. Exp. Brain Res. 1967 2 299-305
- DEMPSEY P.J. and T. COOPER. Supersensitivity to the chronically denervated rat line heart. Am. J. Physiol. 1966 212 1245-1249
- DEPOCAY F. The calorigenic response of cold-acclimated white rats to infused noradrenaline. Can. J. Biochem. Physiol. 1960 38 107-114
- DE SCHRYVER, C., P. DE KRODT and J. LAMONTAGNY. Effect of physical training on cardiac catecholamine concentration. Nature (Lond.) 1967 207 951-953
- DE SCHRYVER, C. and J. MERTENS-STETTINCHEN. Inactivity of exercise and heart tissue on catecholamine content. Pflügers Arch. 1972 338 343-354
- DE SCHRYVER, C., J. MERTENS-STETTINCHEN, J. KOCKEL and J. LAMONTAGNY. Effect of training on heart and skeletal muscle catecholamine concentration in rats. Am. J. Physiol. 1969 217 1599-1592
- DES MEULIS A. and L.F. DODGE. Circulation of phérogène et essai des courbes de noradrénaline sur un artériole (noradrénaline) dans le rat blanc exposé au froid. Can. J. Med. Sci. 1951 29 90-99
- DEVINE G.R. The fine structure of vascular smooth muscle treated with 6-hydroxydopamine. Proc. Nat. Acad. Sci. 1969 66 4-6
- DOUGHERTY, J. and W. De JONG. Diminished receptor response to noradrenaline of the perfused tail artery of pregnant rats. Europ. J. Pharmacol. 1974 23 247-259
- DONALD D.E., D.A. FERGUSON and S.E. MILBURN. Effect of beta-adrenergic receptor blockade on racing performance of Greyhounds with normal and with denervated hearts. Q. J. Exp. Physiol. 1966 51 127-134
- DONALD D.E., S.E. MILBURN and J.T. SHEPHERD. Effect of cardiac denervation on the maximal capacity for exercise in the racing greyhound. J. Appl. Physiol. 1966 19 849-852
- DONALD D.E. and J.T. SHEPHERD. Is total cardiovascular adjustment to exercise in dogs with chronic cardiac denervation. Am. J. Physiol. 1964 207 1225-1229
- DOUGLAS, L.D. and M.J. BRUOT. The sympathetic nervous system and development of experimental renal hypertension. Clin. Sci. 1964 13 342
- DOUGLAS, S. and G. GARDNER. Cephalic and cardio reflex influences on diaphragm function. Am. J. Physiol. 1968 215 1192-1199
- DOUGLAS, S.E. and E. SIEGEL. Baroreceptor and chemoreceptor influences on sympathetic discharge to the heart. Am. J. Physiol. 1963 205 471-479
- EDMOND H., J.G. FLETCHER, R.H. WILCOXSON and R.A. McCABE. The energy expenditure and food intake of individual men. N. J. J. Med. 1955 2 284-300
- EDWARDS A.L. Experimental Methods for Behavioral Sciences 1954. Vol. 1. Reinhold and Wiley. p. 308
- EDWARDS, S.E. and F. FALCK. Adrenergic and cholinergic-containing neurons of the rat. Neuroscience 1968 14 197-203
- ERIKSSON, S., K. ERIKSSON, T. MALMSTROM, S. RICHARDSON and E. STAMFELDT. Sympathetic and pharmacological blockade of lead rats. Acta physiol. scand. 1973 89 283-285
- ERIKSSON, S., K. ERIKSSON and S. MALMSTROM. Physical training, body fluid and autonomic nervous system. Scand. J. Clin. Lab. Invest. 1973 33 251-256
- ERIKSSON, J. The role of the rat in the heart and salivary gland. Am. J. Physiol. 1972 22 73-80
- ERIKSSON, J. Compensatory increase in salivary gland activity in salivary glands and diaphragm muscle of the rat. Acta physiol. scand. 1973 92 525-530
- ELLIOT T.R. and I. TICKETT. Control and metabolism of the suprapulmonary gland. J. Physiol. 1966 24 337-349
- EMERY F.E., R. EMERY and E.L. SCHWARTZ. The effect of prolonged exposure to low temperatures on body growth and on the heart. Am. J. Physiol. 1940 4 17-32

- KANATO L. and O. KANATO Effect of guanethidine on nerve all and small intensely fluorescent cell in sympathetic ganglia of newborn and adult Acta pharmacol toxicol 1971 30 403-416  
 KROKHINA I L. Proliferation dynamics of all elements in ha differentiating mouse myocytes Jei logiya 1968 10 1391-1409  
 KULER U S von Sympathic adrenal activity in physical exercise Medicine and Science in Sports 1974 6 165-173  
 KULER U S von and F. FRANKSON and J. HELLSTEN Adrenaline and noradrenaline output in urine from aorta and bile renal adrenaline only in man. A study Acta physiol scand 1954 31 1-5  
 KULER U S von and S. HELLSTEN Noradrenaline excretion in vascular work Acta physiol scand 1952 26 183-191  
 KULER U S von and P. LINDMARK Improved techniques for the fluorimetric estimation of catecholamines Acta physiol scand 1961 31 348-354  
 KULER U S von and A. FURUKAWA Effect of sympathetic denervation on the noradrenaline and adrenaline content of the spleen, kidney and salivary glands in the beaver Acta physiol scand 1951 24 212-217  
 KYRIAKIS S. B. J. GARNON J. W. HEATH and G. HUNTER Long-term damage to the internal genital organs and their adrenergic innervation in rats following chronic treatment with the antihypertensive drug guanethidine Fertility and Sterility 1972 23 657-667  
 LAMARCA M. G. ORNETTI R. VIOLETTI K. E. KIM and G. SHARPE Effect of chronic adrenal transection of propranolol on the blood pressure and heart weight in experimental renal hypertension Lif Sci 1976 18 967-970  
 LEACH L. and G. D. LEACH The contribution of the sympathetic nervous system to the development and maintenance of experimental hypertension in the rat Br. J. Pharmacol 1970 29 317-324  
 LIEBER J. E. and D. D. ROBERT and I. J. KOPPEL Noradrenaline metabolism in hypertrophied hearts Nature (London) 1965 207 951-953  
 LINDHOLM W. W. Sensitive sensitivity in smooth muscle: a introduction and historical perspective Fed. Proc 1973 34 1968-1970  
 LINDHOLM W. W. P. R. ORQUILLA D. A. TAYLOR and D. P. WESTWALL, Electrophysiological correlations with postjunctional supersensitivity Fed. Proc 1973 34 1961-1964  
 LINDHOLM W. and U. S. von KULER Selective activation of noradrenaline and adrenaline producing cells in the cat adrenal gland by hypothalamic stimulation Circ. Res 1954 2 191-195  
 LINDHOLM W. G. GILBERT and G. THULESIUS Adaptive structural changes of the vascular walls in hypertensive and their relation to the control of the peripheral resistance Acta physiol scand 1958 44 235-272  
 LINDHOLM W. M. HALLBACK Y. LUNDGREN and L. WEISS Background of increased flow resistance and vascular reactivity in spontaneously hypertensive rats Acta physiol scand 1970 80 93-106  
 LINDHOLM W. M. HALLBACK Y. LUNDGREN and L. WEISS The effects of immunosympathectomy on blood pressure and vascular reactivity in normal and spontaneously hypertensive rats Acta physiol scand 1972 86 512-523  
 LINDHOLM W. M. Behaviour and of releasing catecholamines Brain Research 1971 21 241-262  
 LINDHOLM W. M. and H. RANOLSKY Effects of thyroid hormones on arterial noradrenaline organ systems I. Handbook of Physiology 1974 Section 7 Endocrinology Vol III Thyroid, pp 425-446 Eds M. A. Cren and D. H. Solomon American Physiological Society Washington  
 LINDHOLM W. M. J. G. PAPP and E. M. WADSWORTH WILLIAMS The effect of altered thyroid status on arterial resistance in the rat J. Physiol (London) 1970 207 357-369  
 LINDHOLM W. M. and L. CHALAY Effect of regular muscular activity on adrenocortical function in the rat J. Sports Med Phys Fitness 1962 2 207-211  
 LINDHOLM W. M. L. CHALAY and G. CSÁKVÁRY A study of the stress reaction elicited by muscular exertion in trained and untrained man and rat Acta physiol Scand 1969 24 345-370  
 LINDHOLM W. M. L. CHALAY and G. CSÁKVÁRY and T. ZILLES Effect of muscular exertion on the reaction of the pituitary-adrenocortical axis in trained and untrained rat Acta physiol Scand 1968 33 35-438  
 LINDHOLM W. M. and I. ÖRTENGREN and O. SJÖSTRAND Effect of training on the release of fatty acids and adrenaline in muscle and on lipolysis in adipose tissue in vivo Acta physiol scand 1972 84 166-177

- FORDER R. R. KAMM and A. LAMBERT Der Einfluss von Hypoglykämie und Muskelarbeit auf die Elektrolytenkonzentration der Nebennieren rindenhormone *Ischvys med Vrach* 1954 84 304-305
- FURUKAWA R. The classification of adrenergic receptors (adrenergic receptors) An evolution from the standpoint of receptor theory In Calcium Handbook of Experimental Pharmacology 1972 23 pp 283-335 Eds R. Blumberg and S. Monodell Springer Verlag Berlin Heidelberg New York.
- FURUKAWA, C.M. R. J. LINDER and R.M. SHON Chronotropic and inotropic effects on the dog heart of stimulating the afferent cardiac sympathetic nerves J. Physiol. 1973 230 137-153
- GARRETT R. J. T. DAYAN D. BURGESS J. CHERRY and M.L. HENSHAW Prolonged effects of chronic guanethidine treatment of the sympathetic innervation of the penis of rat Med. J. Australia 1971 2 207-208
- GARCIA R. and J.M. JENNIFER Phaeochromocytoma masquerading as cardiomyopathy Amer. J. Cardiol. 1972 29 548-571
- GEORGE A. and B. POLLOCK The involvement of corticosteroids in the supersensitivity produced in the rat aortic myocytes by morphine withdrawal thyroidectomy or single dose of reserpine J. Pharmacol. Exp. Therap. 1975 193 390-398
- GEORGE A.L. Relationship between baroreceptors and vascular work in determining aortic flow Cardiac Hypertrophy pp 39-53 Ed R.R. Alpert Academic Press New York and London, 1970
- GOLDMAN R. and D. JACOBOWITZ Correlation of norepinephrine content with observations of adrenergic nerves after single doses of 6-hydroxydopamine in the rat J. Pharmacol. Exp. Therap. 1971 176 119-133
- GOLDMAN R.M. and D.C. HARRISON The effects of hypoxia and hypercarbia on myocardial catecholamines J. Pharmacol. Exp. Therap. 1970 174 307-314
- GOLDSTEIN M. S. ARACOSTE A. F. BENTLEY V. S. OWEN and S. MASATANI Studies of changes in the striatum in monkeys with nigral lesions. The disposition of levodopa and metabolites of 6-dopamine and 14C serotonin in the striatum J. Neurochem. 1969 16 645-653
- GORDON R. J. V. KIDD A. SJÖQVIST and S. UNGERLID Increased synthesis of norepinephrine in the rat heart on electrical stimulation of splanchnic ganglia Med. Pharmacol. 1966 2 610-613
- GORDON R. S. SPECTOR, A. SJÖQVIST and S. UNGERLID Increased synthesis of norepinephrine and epinephrine in the intact rat during exercise and exposure to cold J. Pharmacol. Exp. Therap. 1966 193 440-447
- GOSLIN R. The Hyperkinetic Bear Syndrome J. Amer. Med. Assoc. 1962 182 823-829
- GOSLIN R.J. Adaptive growth of the heart In Cardiac Hypertrophy 1971 pp 1-10 Ed R.R. Alpert Academic Press New York and London
- GRANTING A. R. S. MALIK and R.M. HILKES Effects of thyroid hormones on growth differentiation and development In Handbook of Physiol 1974 Section 7 Endocrinology Vol III Thyroid pp 377-389 Eds M.A. Greer and D.R. Solomon American Physiological Society Washington
- GRUBB, A. F. R. KNOTH and W. V. WHITEHEAD Properties of myocardium in cardiomegaly Circ. Res. 1963 12 118-126
- HARRISON, G. W. HARTLEY and A. HARRISON Effects of surgical and chemical adrenergic denervation on vascular responses Proc Symp Physiol Pharmacol Vasc Neuroeffector System Interlake 1966 pp 141-159 Basel Karger
- HARRISON, G. W. HARTLEY and R. THOMAS Chemical sympathectomy of the rat with 6-hydroxydopamine J. Pharmacol. Exp. Therap. 1966 170 90-61
- HARRISON, J. L. R. BARTLEY and R. SALIN Arterial noradrenaline concentration during exercise in relation to the relative work level Scand. J. Lab. Invest. 1970 24 337-342
- HARRISON, J. and T. PALMFOR The effect of nerve stimulation on the uptake of noradrenaline by the adrenergic nerve terminal Acta Physiol. Scand. 1969 22 28-32
- HARRISON, F. R. Active spreading activity and body temperature regulation in the rat Am. J. Physiol. 1947 213 1289-1292
- HALL, O. C. E. HALL and E. OGDEN On the hypertrophy in experimental hyperosmia and its regression following establishment of normal blood pressure Am. J. Physiol. 1933 174 175-178
- HALLIDAY M. Interaction between central neurogenic mechanisms and changes in cardiovascular design in primary hypertension Acta Physiol. Scand. 1975 Suppl. 424 1-59
- HARRINGTON, E. R. LEVI-MONTALCINI R.A. ROYCE and F. SJÖQVIST Noradrenaline in immunosympathectomized rat Int. J. Neuropharmacol. 1965 4 91-95



- KIMBLE, J. and I. M. MAZURKIEWICZ-KULICKI Unchanged sensitivity noradrenaline in isolated  $\text{HE}$  same from cold-acclimated rats *Can J Physiol Pharmacol* 1970 **48** 657-660
- KIMURA, T. Über den Einfluss der Körperkühlung bei Anstrengung f. d. Herz *Mitt. d. med. Fak. d. Kaiserl. Univ. Tokyo* 1919 **22** 189-243
- KJÖRSTEDT, A., B. JOHNSON, M. LEVANDER, M. LINDBERGH, T. SJÖSTRAND and G. STENH. Low physical working capacity in suspected heart disease due to inadequate adjustment of peripheral blood flow (Vasoregulation or Anemia) *Acta Med. Scand.* 1957 **158** 413-434
- KJÖRSTEDT, A., B. JOHNSON, M. LEVANDER, M. LINDBERGH, M. MOSEFELDT, T. SJÖSTRAND and G. STENH. Physical training of patients with Vasoregulation or Anemia *Acta Med. Scand.* 1957b **158** 437-446
- KULICKI, I. M., K. CHEDDER and G. KRONBERG Ueber die sympathicomimetische pressorische Prinzip des Noras ("Norosympathin") *Arch. Exp. Path. Pharmacol.* 1947 **204** 228-243
- KULICKI, I. M. and D. SHARPE The distribution of catecholamines in vertebrates as in *Handbook of Experimental Pharmacology* XXXIII. Catecholamines 1972 pp 110-185 Eds E. Blaschko and E. Muscholl Springer Verlag Berlin Heidelberg New York.
- KURITA, M., W. V. JUDY and L. D. CARLSON Effects of drenaline and noradrenaline on an arterial in cold- and warm-blooded rats *J. Appl. Physiol.* 1962 **17** 754-758
- KUWAMOTO, S. Elektrokardiographische Untersuchungen der Amsterdamer Olympiadokampfer *Arbeitsphysiologie* 1929 **2** 61-75
- KWAT, W. Morphologische und physikalische Untersuchungen an den Geweben eines Lebertrainings und nach dem Training *Virchows Archiv* 1951 **220** 157-237
- KWAT, I. and P. A. VAN ELLIEN The central hypotensive action of sympathamine applied to pharmacological chloroform and fenflurine *J. Pharm. Pharmacol.* 1972 **24** 432-458
- KWAT, A. C. L. and L. D. CARLSON Role of drenaline and noradrenaline in hemolysis regulation of hemoglobin production *Am. J. Physiol.* 1957 **190** 243-246
- KWAT, A. C. L., C. W. FUN, K. M. LI and K. W. TI Circulatory and metabolic effects of noradrenaline in cold-adapted rats *Can. J. Physiol. Pharmacol.* 1966 **44** 1805-1812
- KWAT, W. M., C. C. FORTNA and R. PASCHKE Growth hormone secretion and its role in muscle exercise in rats *Endocrinology* 1965 **150** 1051-1053
- KWAT, D. A., J. D. M. PEARSON and D. F. SHARPE Striatal metabolism of dopamine in mice made aggressive by isolation *J. Neurochem.* 1975 **24** 1151-1154
- KWAT, R. Pre- and post-synaptic metabolism in the nerve cell during growth and function *Acta Physiol. Scand.* 1943 **6** Suppl. 17 1-134
- KWAT, R. Dynamic aspects on the neuro-glial relationship I *The Neuron* 1967 pp 179-219 Ed. M. Hyden Elsevier Amsterdam London New York.
- KWAT, A. J., J. P. BARKETT and L. PROCTOR centrally mediated peripheral hypotensive effect of methylcholine *J. Pharm. Exp. Therap.* 1970 **172** 393-399
- KWAT, A. J. and D. D. BOWYCASTLE The effect of exposure to heat and cold upon the level of catecholamines and 5-hydroxytryptamine in the rat *Can. J. Physiol. Pharmacol.* 1967 **45** 733-743
- KWAT, L. L. and J. GLOWINSKI Regional studies of catecholamines in the rat brain II Rate of turnover of catecholamines in various brain regions *J. Neurochem.* 1966 **13** 671-682
- KWAT, L. L., J. GLOWINSKI and J. AXELROD The physiological disposition and metabolism of norepinephrine in the sympathetically denervated animal *J. Pharmacol. Exp. Therap.* 1966 **121** 273-284
- KWAT, L. L., J. GLOWINSKI and M. A. SIMMONS Differences in the uptake, storage and metabolism of ( ) and ( )-noradrenaline *Br. J. Pharmacol.* 1971 **2** 843-855
- KWAT, L. L. and F. J. SALT Isolation of catecholamine uptake by nerve terminals in the isolated rat heart *Br. J. Pharmacol.* 1970 **40** 328-330
- KWAT, K. K., K. KARIYA and S. FUJIMOTO Effects of compounds affecting the dopaminergic mechanism on cell growth and division of *Tetrahymena pyriformis* W *J. Pharm. Pharmacol.* 1969 **12** 273-281
- JACKSON, C. M. The effects of malnutrition and malnutrition upon growth and development 1925 pp 227-278 *Blak.* on Phil. delphic
- JACOBOWITZ, D. Neurochemical studies of the relationship of chromaffin cells and dopaminergic nerve fibers to the autonomic ganglia of several species *J. Pharmacol. Exp. Therap.* 1967 **158** 227-240
- JACOBOWITZ, M. Long-term effects on peripheral dopaminergic nerves of 5-hydroxydopamine and its metabolites *J. Chem. Biol. Physiol.* 1975 **1** Catecholamine Research. Vol. 1 pp 153-162 Ed. E. Jönsson T. Malmberg Kluwer Academic Publishers Company Amsterdam Oxford

- JACOBOWITZ D. T. COOPER and H. B. BARKER: The toxicologic and chemical studies of the local anesthetic E. dromargi and cholinergic nerves in normal and denervated cat hearts. Circ. Res. 1967 20 289-298
- JARISKY L. R., BARTUKOVA J., KOCKOVA J., MELISAR and E. ZEISBERGER: Interspecies differences in cold adaptation and non-shivering thermogenesis. Fed. Proc. 1969 28 1053-1054
- JARISKY L. R., BARTUKOVA J. and E. ZEISBERGER: Acclimation of the white rat to cold: norepinephrine thermogenesis. Physiol. Bohemoslov. 1967 16 344-372
- JARISKY L. and J. E. BARTY: Cardiac output and organ blood flow in warm- and cold-acclimated rats exposed to cold. Canad. J. Physiol. Pharmacol. 1968 46 653-659
- JERGEN-HOLM J. and P. JULI: Ultrastructural changes in the rat superior cervical ganglion following prolonged guanethidine administration. Acta pharmacol. toxicol. 1971 30 308-320
- JOHNSON E. M., E. CANTON and J. R. DOUGLAS: Biochemical and functional evaluation of the yolk sac produced by the administration of guanethidine to newborn rats. J. Pharmacol. exp. Ther. 1975 193 503-512
- JOHNSON R. H., D. M. PARK, M. J. HENRIE and W. R. SULAIMAN: Hormonal responses to exercise in long cycle rats. J. Physiol. 1974 241 23-25p
- JORGE S. E. de and W. ROSENTHAL: Über den Cholesteringehalt der Nebennieren erkrankter Tiere. Acta brev. neerland. physiol. 1933 3 86-88
- KALISER S.: Survival potential of responses to sympathomimetic amine, in sorted tripe. Br. J. Pharmacol. 1969 36 582-593
- KÄRKL H. T.: The urinary excretion of norepinephrine and epinephrine in different age groups: its diurnal variation and the effect of muscular work on it. Acta physiol. scand. 1954 29 Suppl. 132
- KAST E.: Die Wirkung von Adrenalin und Adrenalin auf die Herz-Sportleistung. Wien. Klin. Wochenschr. 1924 37 212-214
- KEATINGE W. R.: The effect of repeated daily exposure to cold and of improved physical fitness on the metabolic and vascular responses to cold. J. Physiol. (Lond.) 1961 137 209-220
- KETTY S. S., F. JAVOY, A. M. THIRRY, L. JULOU and J. GLONINSKI: A sustained effect of leucocortisol shock on the turnover of norepinephrine in the central nervous system of the rat. Proc. Nat. Acad. Sci. 1967 26 1249-1254
- KIRPEKAR S. M., P. CERVOSSI and R. P. FURCHGOTT: Catecholamine content of the adrenal medulla following procedures on nitric oxide to norepinephrine. J. Pharmacol. exp. Ther. 1962 132 180-190
- KLINKE O. and E. STÖCKER: Die DNS-Synthese im Ratembirn: Die Funktion des Lebensalters. Anterodigraphische Untersuchungen an der Thyroid. Experientia 1966 24 167-168
- KLINSMAN G. I.: Catecholamine levels and dopa-decarboxylase activity in peripheral organs and adrenal glands in the rat of immunosympathectomy. J. Pharmacol. exp. Therap. 1965 148 14-21
- KNUTT C. A., D. B. DILL and W. MEYER: Training and its effect on man: rest and work. Am. J. Physiol. 1942 136 148-154
- KOPIN I. J., G. R. SACESE, K. R. KRAUS and V. K. WEISS: Selective release of newly synthesized norepinephrine from the pleon during sympathetic nerve stimulation. J. Pharmacol. exp. Ther. 1966 161 271-278
- KORLECKY R. and H. KILBICK: Effect of thyroxine on growth and function of caecal mass. J. Caecal Hyperrophy 1971 Ed. H. R. Alpert pp. 53-64. Academic Press Inc. New York and London
- KUHL F.: Experimentelle Studien über Pharmakologie und Arbeit. Archiv. Exptl. Path. u. Pharmacol. 1906 22 288-303
- KÜLS F.: Über den Einfluss der Bewegung auf den von Handen und Brustkammern regulierten. Deutsche Med. Wochenschr. 1912 1 1916-1920
- KVETNAJSKY R., G. P. GOVINTZ, V. K. WEISS and I. J. KOPIN: Effect of hypophysectomy on immobilization-induced release of tyrosine hydroxylase and phenylthiohydrazine-N-methyltransferase in the rat adrenal gland. Endocrinology 1970 87 1323-1329
- KVETNAJSKY R., G. P. GOVINTZ, V. K. WEISS and I. J. KOPIN: Catecholamine synthesis in the rat adrenal gland during exposure to cold. Am. J. Physiol. 1971 220 928-931
- KVETNAJSKY R., G. P. GOVINTZ, K. WEISS and I. J. KOPIN: Enhanced synthesis of adrenal dopamine  $\beta$ -hydroxylase induced by open field immobilization. Mol. Pharmacol. 1971b 1 81-86
- KVETNAJSKY R. and L. MIEHLA: Adrenal and urinary catecholamine levels during depletion of adrenal immobilization. Endocrinology 1970 87 738-743

- LALANNE J L, K. SCHMITT and R. SCHMITT Sex l adonisme du l type tension provoquée par l adrénaline chez l t. Archs Pharmacodyn Ther 1966 163 444-464
- LAMPRICHT F R, EICKELMAN M R, THOA M R, WILLIAMS and I J ROFIN Rat fighting behaviour serum dopamine- $\beta$ -hydroxylase and hypothalamic tyrosin hydroxylase Science 1972 177 1214 1215
- LANCE AUBREY E. Metabolism and circulatory aspects of tolerance to cold effected by physical training Acta physi 1966 19 144-151
- LAVERTY R. and R.L. PHILLAN Effects f 6-hydroxydopamine on noradrenaline storage and uptake in the rat Proc Uni Orange med School 1966 47 18 19
- LEHMAN J Effect f adrenalectomy noradrenaline and chlorpromazine on blood pressure f normal and cold adapted rats Prog Soc Exptl Biol Med 1960 105 109 111
- LEHMAN J and M. FOULLOT Importance f noradrenaline in cold adaptation Am. J. Physiol 1964 207 853-856
- LEHMAN J J WILLIAMS and G. VACHON Beta receptor sensitization by repeated injection f isoproterenol and by cold adaptation. Am. J. Physiol 1972 222 1043-1046
- LEHMAN J A, VILLEMARTE and J WILLIAMS Simultaneous sensitization to norepinephrine and cardiovascular effects f noradrenaline Arch Intern Physiol Mechan. 1969 77 731 740
- LEMO J Catecholamine production and release in exposure and colimation to cold Acta Physiol 1961 22 Suppl 183 1 101
- LENNER, B and E. SALLER Difference i the turnover f Noradrenaline in Rat Heart during Day and Night Neuro-Schmidberg Arch Pharmacol 1973 278 107 109
- LENNER, B and E. SALLER, Influence f light and darkness on the turnover f noradrenaline in the rat heart Neuro-Schmidberg Arch Pharmacol 1974 282 75-84
- LEON A.B and C.M. KLOOR, Effects f exercise and its cessation on the heart and its blood supply J. Appl. Physiol 1960 24 485-490
- LEON A.B W.D. ROBERT H. SPIERT Z.B. WIGGAN and A.W. WINKLESDORF Sea t norepinephrine level fte exercise training in the rat Chest 1973 67 341 343
- LEVI R. and E.W. MATHIAS Effects f trans on brain norepinephrine Fed. Proc 1942 21 334
- LEVI-MONTALCINI R. and P.U. ANGELETTI Noradrenaline and monoamine oxidase content in immunosympathectomized animal Int J Neuropharmacol 1962 1 161 164
- LEVI-MONTALCINI R. and P.U. ANGELETTI Immunosympathectomy Pharmacol. Rev 1966 18 619 628
- LEVI-MONTALCINI R. and B. BOOKER Destruction f the sympathetic ganglia by an antiserum t nerve-growth protal Prog. Biol. Acad. Sci. Wash 1960 46 324 331
- LEVITT M., SPECTOR, A. SJOGREN and E. EDENFELD, Elimination f the rate-limiting step in norepinephrine synthesis in the perfused guinea-pig heart J. Pharmacol. Exp. Ther 1963 144 1-8
- LEWIS G.F. Physiol glial mechanisms controlling secretory activity f adrenal medulla In Handbook f Physiology 1973 Section 7 Volume VI Adrenal Gland pp 309 319 American Physiological Society
- LIN Y-C and H.M. HORWITZ Antisocial nervous control f rillac f frequency in the mouse a-trial ed rat J. Appl. Physiol 1972 33 796 799
- LINDBQVIST A and B. OLCK The fiber in response dial vasculature i on some forms f experimental cardiac hypertrophy Acta sci p etiol vi scand 1972 Section A. 80 329 340
- LOMBE R F, E. KENN and B. BARTHOLOMI Degener on and regeneration f adrenergic nerves in the rat blood vessel i xl and rille f the rat f 6-hydroxydopamine injection J. Neurocytol 1975 4 157 176
- LOUIS W.J. SPECTOR E. TAMEI and A. SJOGREN Synthesis and turnover f norepinephrine i the heart f the mouse anoxically hyperoxia Surg. Exp 1960 25 85-91
- MATTHE L. Presence f -ne hyl-dopa in the heart and brain f guinea pigs treated w -ne hyl tyrosine Life Sci 1965 2249 2256
- MALIK A.B T. ME R.O. G. KANE and B. CHEN Cardiac performance in ventricular hypertrophy induced by pressure and volume overloading J. Exptl. Physiol 1974 22 847-874
- MALMPOSS T and C. SACHS Degener on f adrenergic nerves produced by 6-hydroxydopamine Europ Pharmacol 1968 3 89-92
- MALVALDI G., MEDACCI and M.P. VIOGA-MACCHI MI cases in the adrenal medullary cells. Experientia (Basel) 1968 24 75-87



- SWEDIN E. Comparison of the effects of  $\alpha$ -methyltyrosine on the noradrenaline stores of different peripheral organs of the rat Life Sci 1970 9 1249-1259
- SWEDIN G. Studies on neurohumoral mechanisms in the rat and guinea pig via deferens Acta physiol scand 1971 93 Suppl 369 1-34
- TIJUN A K. and W. de JONGH Time course of decrease in blood pressure and in blood pressure response to vasopressor agent during pregnancy in the rat Europ J Physiol (Pflüger Arch) 1973 341 197-200
- TAKEATSU T and C. KAMEI Cardiac hypertrophy in spontaneously hypertensive rat IN Spontaneous Hypertension pp164-172 Ed K. Okamoto Igaku Shoin Ltd Tokyo and Springer Verlag Berlin Heidelberg New York 1972
- TAN E.M. M.K. WARSON and E.P. RICHTER Swimming time of rats with relation to water temperature Yed. Proc 1954 13 150-151
- TAYLOR E.M. and R. LAVEY The wet bolus of tritiated dopamine in regions of rat brain in vivo II J. Neurochem 1969 16 1367-1371
- THARP G.D. and R.J. NUCK, Adrenal adaptation to chronic exercise J. Appl. Physiol 1974 37 720-722
- THIRRY A.M. G. BLANC and J. GLOUTSKI Effects of stress on the disposition of catecholamine localized in various intraneuronal storage forms in the brain of the rat J. Neurochem. 1971 19 449-461
- THOMAS E. Induction of tyrosine hydroxylase in peripheral and central adrenergic neurons by cold exposure Life Sci 1970 22 861-862
- THOMSEN K. Surgical immunological and chemical sympathetomy Their application in the investigation of the physiology and pharmacology of the sympathetic nervous system. IN Catecholamines, Handbook of Experimental Pharmacology 1972 33 pp 813-844 Ed E. Blaschko and E. Moschell Springer Verlag Berlin Heidelberg New York
- THOMSEN K. U. OTTEN and F. GIESCH Trans-synaptic regulation of tyrosine hydroxylase IN Frontiers in catecholamine research pp179-185 Ed E. Lefkowitz and S. K. Snyder Pergamon Press Inc New York, Toronto Oxford Sydney Academic Press 1973
- THOMSEN K. and J.P. TRANIER Chemical sympathetomy by selective destruction of adrenergic nerve ending with 6-hydroxydopamine Neuro-Schmeldeberg Arch Pharmak exp Path 1966 261 271-288
- THOMAS R.M. and A.T. MILLER J. Adaptation to forced exercise in the rat Am. J. Physiol 1956 193 350-354
- THOMSEN U. Trainingsversuche an Ratten. III. Histologische Beobachtungen an Herz und Skelettmuskeln Arbeitsphysiologie 1954 33 9 339-370
- THOMAS R.M.G. and L.A. STUCKER Effects of phenylephrine on ex vivo tyrosine of liver regeneration in partially hepatectomized rat Molham, Pharmac 1973 22 93-100
- TIPTON C.M. Training and bradycardia in rat Amer. J. Physiol 1965 209 1089-1094
- TIPTON C.M. and B. TAYLOR Influence of tropine on heart rate of rats Am. J. Physiol 1965 208 480-484
- TIPTON C.M. and T.-K. TCHENG Influence of physical training on corticosterone secretion and pituitary hormone on the mean weight of hypophysectomized rats Pflüger Arch 1971 322 103-112
- TRANIER J.P. and K. THOMSEN Ultrastructural changes of the sympathetic nervous system during the development of the rat before and after 6-hydroxydopamine Neuro-Schmeldeberg Arch Pharmak exp Path 1967 257 343-344
- TRANIER J.P. and K. THOMSEN An electron microscopic study of selective acute degeneration of sympathetic nerve terminal after administration of 6-hydroxydopamine Experientia 1968 24 135-136
- THOMSEN K. U. Modification of the effects of tyrosine by various agents and procedures J. Pharmacol Exp Therap 1961 134 8-17
- THOMSEN K. U. Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines Pharmacol. rev 1966 18 629-640
- THOMSEN K. U. Factors influencing the concentration of catecholamines in the nervous system IN Catecholamines, Handbook of Experimental Pharmacology 1972 33 pp 774-781 Eds E. Blaschko and E. Moschell Springer Verlag Berlin Heidelberg New York
- TUNNICLIFFE P.  $\beta$ -adrenergic receptor blocking drugs in hyperthyroidism Drugs 1974 7 48-54
- UNO C. E. MARCACKI and A. LINDQVIST The sympathetic myocardial innervation in various forms of experimental cardiac hypertrophy Arch Pathol (Lab) 1973 31 364-375



- WONG W K I O FLYNN and T INOUE Micromethods for measuring phenylalanine and tyrosine in serum Clin. Chem 1964 10 1098-1104
- WURTMAN R.J Control of epinephrine synthesis in the adrenal medulla by the adrenal cortex: hormonal specificity and dose-response characteristics Endocrinology 1966 79 608-614
- WURTMAN, R.J and J AXELROD Control of enzymatic synthesis of norepinephrine in the adrenal medulla by adrenal cortical steroids J. Biol. Chem 1966 241 2301-2305
- YAMORI Y Contribution of cardiovascular factors to the development of hypertension in spontaneously hypertensive rats Jap. Heart J 1974 15 184-194
- YAMORI Y The immunosympathectomized animal: a valuable tool in physiological and pharmacological research J Physiol (Lond) 1965 177 35P-37P
- YAMORI Y, L. HIRK and B. CALLINGHAM, Morphological, biochemical and functional changes in the sympathetic nervous system of rats treated with nerve growth factor Cell Tissue Res (Lond) 1965 306 1270-1272





ACTA PHYSIOLOGICA SCANDINAVICA  
Supplementum 478

Human Power  
at  
Subnormal Body Temperatures

by  
Ulf Bergh

STOCKHOLM 1980



ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 478

FROM THE DEPARTMENT OF PHYSIOLOGY III  
KAROLINSKA INSTITUTET  
STOCKHOLM SWEDEN

Human Power  
at  
Subnormal Body Temperatures

by  
Ulf Bergh

STOCKHOLM 1980





This thesis is based on the following papers

- I MADEL E R HOLMÉR I BERGH U ASTRAND P -O & STOLWIJK J A J 1973 Thermoregulatory shivering during exercise *Life Sci* 13 983 989
- II BERGH U EKBLOM B HOLMÉR I & GULLSTRAND L 1978 Body temperature response to a long distance swimming race *Swimming Medicine IV* (Eds B O Eriksson & B Furberg) pp 342 344 University Park Press Baltimore
- III DAVIES M EKBLOM B BERGH U & KANSTRUP I L 1975 The effect of hypothermia on submaximal and maximal work performance *Acta Physiol Scand* 94 201 202
- IV BERGH U HARTLEY H LANDSBERG L & EKBLOM B 1979 Plasma norepinephrine concentration during submaximal and maximal exercise at lowered skin and core temperatures *Acta Physiol Scand* 106 383 384
- V BERGH U & EKBLOM B 1979 Physical performance and peak aerobic power at different body temperatures 1979 *J Appl Physiol Respirat Environ Exercise Physiol* 46 885-889
- VI BERGH U & EKBLOM B 1979 Influence of muscle temperature on muscle strength and power output in human skeletal muscle *Acta Physiol Scand* 107 33 37
- VII BERGH U BLOMSTRAND E & ESSÉN B 1980 Human anaerobic energy yield and muscle metabolites at subnormal body temperatures Manuscript

In addition some hitherto unpublished results will be presented

The papers are referred to in the text by their Roman numerals



## C O N T E N T S

ABBREVIATIONS AND SYMBOLS	6
INTRODUCTION	7
METHODOLOGICAL ASPECTS	
Methods	9
Subjects	11
Procedure	11
Statistics	11
Comments	11
RESULTS AND DISCUSSIONS	
Rectal temperature after long distance swimming	15
Aerobic power	16
Rest and submaximal exercise	16
Exhaustive exercise	20
Anaerobic power	24
Muscular strength	27
Physical performance	28
SUMMARY	33
ACKNOWLEDGEMENTS	35
REFERENCES	36

## Abbreviations and symbols

The abbreviations and symbols used in the text and figures are with minor exceptions adapted to the guidelines devised by the Glossary Committee of the International Union of Physiological Sciences (1973a b). The units of the SI system are used.

ABP	arterial blood pressure
ATP	adenosine triphosphate
a-v difference	arterio-venous difference
°C	degree Celsius (Centigrade)
CP	creatine phosphate
G-6 P	glucose-6-phosphate
HR	heart rate
peak HR	highest HR measured in a particular situation
J	joule
LA	lactate
NE	norepinephrine
peak oxygen uptake	highest oxygen uptake measured in a particular situation
maximal oxygen uptake	highest oxygen uptake obtained irrespective of body temperature during exercise with large muscle groups breathing normal air at an air pressure equal to sea level
maximal aerobic power	
Q	cardiac output
RPE	rated perceived exertion
SV	stroke volume
T <sub>c</sub>	core temperature
T <sub>es</sub>	esophageal temperature
T <sub>m</sub>	muscle temperature
T <sub>re</sub>	rectal temperature
T <sub>sk</sub>	mean skin temperature
T <sub>w</sub>	water temperature
W	watt
WT	work time
Ventilatory equivalent	pulmonary ventilation/liter of oxygen uptake

## INTRODUCTION

Man is homeotherm which means that the body temperature is kept at a nearly constant level practically independent of the environmental temperature. This enables man to maintain a high level of functional capacity in a great variety of environmental conditions though the metabolic cost of this privilege is relatively high. On the other hand man is unable to escape very far from homeothermy without approaching lethal body temperatures.

A constant body temperature will be maintained only if heat production and heat gain are balanced by an equally large heat loss. To ensure homeothermy in conditions involving a change in the ambient temperature and/or the level of physical activity one must therefore be able to change any of these variables considerably. This can be achieved in two distinctly different ways: 1) physiological thermoregulatory responses such as increased metabolic rate (mainly by shivering), sweating and changes in the peripheral blood flow; 2) behaviour e.g. clothing, shelter, external heating. Behaviour has greatly extended the range of temperature in which man can survive and live as a permanent resident. This refers mainly to cold since the physiological thermoregulation seems to be predominantly geared towards heat dissipation. Man can be considered to be a tropical animal.

Notwithstanding these powerful means of maintaining independence of the environmental temperature, homeothermy is in fact only partial and furthermore it is limited as pointed out by Burton and Edholm (1955). Partial because many tissues e.g. skin and muscles display a considerable variation in temperature. Actually only the body's central parts (the core i.e. CNS, heart, viscera) have an almost constant temperature. The largest temperature gradients as well as the greatest temperature variation in a given tissue occur during cold exposure. Homeothermy is limited in that man cannot withstand the more severe climates of this planet without behavioural responses. Inasmuch as behavioural protection cannot always be ensured or sometimes even voluntarily abandoned, man has to face the risk of losing the homeothermy battle with consequences that range from moderate hypo- or hyperthermia to lethal temperature levels. Thus cold exposure can produce hypothermia of such a degree that many body functions are seriously affected and it may even

cause death (for refs see Keatinge 1969 MacLean & Ernstie-Smith 1977) In these instances the exposure is usually accidental e.g. shipwrecks a fall from a boat rapid changes in weather e.g. during hikes in the mountains Voluntary exposure to less severe conditions is not unusual Activities involving water immersion are of special interest mainly because the rate of heat loss from the body can be very high in water

Thus it is of interest to know the body temperature response to such conditions Furthermore it is important to be aware of the price (in terms of the effect on optimal function) of deviations from homeothermy

Previous studies on humans have predominantly been concerned with resting conditions and/or submaximal exercise Few data are available on exhaustive exercise Furthermore most investigations have been conducted with the subjects immersed in water which has some inherited problems (cf Methodological aspects) Studies relevant to this topic will be discussed below in connection with the results of the present studies

Present knowledge in this field is limited concerning

- 1) the incidence and extent of body cooling during exposure to moderately cold ( $18-20^{\circ}\text{C}$ ) water
- 2) the possibilities of separating the eventual effects of  $T_{\text{es}}$ ,  $T_{\text{m}}$  and  $T_{\text{sk}}$  on various factors during maximal exercise
- 3) the possibilities of explaining the observed effects of body temperature on HR oxygen uptake and performance
- 4) quantifying the effects of changes in various body temperature on functions of importance for the physical performance

Thus the object of the present series of experiments was to extend present knowledge of the abovementioned problems at a range of subnormal body temperatures where there are no symptoms of severe hypothermia and/or frostbite

## METHODOLOGICAL ASPECTS

Most previous studies have been performed with the subjects immersed in water of different temperatures which has some definite drawbacks. In water there is often a co-variation between  $T_c$  and  $T_{sk}$  making it difficult to separate their relative importance. As the temperature of water affects its physical properties external work might change which would complicate the measurements of performance. Swimming is technically rather difficult and distal parts cool more quickly than the rest of the body. Those two factors together might induce a limitation to performance before other factors are stressed to their maxima. Furthermore swimming does not induce a maximal load on aerobic power (Holmér 1972).

Therefore in order to reduce these problems in the present series of experiments it was considered necessary to

- 1) use technically uncomplicated types of exercise
- 2) use water only as a cooling medium and to perform the experiments in air
- 3) use combined arm- and leg exercise when the object was to stress the aerobic power to its maximum (cf Bergh et al 1976)

Low body temperatures were produced by immersion in cold water either the whole body (I III IV V) or just the legs (VI VII). Different levels of skin temperatures were achieved by regulating the ambient temperature at suitable levels sometimes in combination with exposure of the subject to increased air velocity and artificial skin wetness.

### Methods

Only a summary of the methods used is given here. For details the reader is referred to the separate reports.

Bicycle exercise was performed in an upright position on mechanically braked ergometers (Monark) either as leg exercise (I VI VII) or as combined arm and leg exercise (III IV V). In the latter case the ratio between arm and leg work rates was 1/4 which produces the same maximal oxygen uptake as treadmill running (uphill) (Bergh et al 1976).



The exhaustive exercise tests on a bicycle ergometer were performed for 15-8 min at a constant pedal rate (60 revolutions  $\text{min}^{-1}$ ) and the maximum work time was used as a measure of physical performance. The point of exhaustion was considered to have been reached when the subject was unable to keep up the pace for 5 consecutive revolutions (III-IV-V-VII).

Oxygen uptake was calculated from expired air samples collected in Douglas bags. Volume was measured in a balanced spirometer. Gas samples were analyzed for  $\text{CO}_2$  and  $\text{O}_2$  content by the Haldane or the Sholander technique (I-III) or by the use of a mass spectrometer (MGA 200 UK) (Hallböök et al 1978) (IV-V-VII).

Oxygen deficit was derived by subtracting the total oxygen uptake from the calculated oxygen requirement of the actual rate of work (Åstrand & Rodahl 1977) (VII).

Heart rate was calculated from ECG-records.

Blood lactate concentrations were determined by the Baker Summerson method as modified by Ström (1949) (III-IV-V-VII).

Arterial blood pressure was measured by a sphygmomanometer (IV).

Muscle biopsies were obtained with the percutaneous needle biopsy technique (Bergström 1962) (VI-VII).

Muscle samples were analyzed with methods described by Essén (1978) (VI-VII).

Plasma norepinephrine concentration was measured as described by Lake et al (1978) (IV).

Maximal muscle strength in the knee extensors was measured on an isokinetic dynamometer (Cybex II, Lumex Inc, New York) (VI).

Sprinting performance was defined as the shortest time in which 20 revolutions (8.5 kJ) could be performed on a bicycle ergometer (VI).

Jumping performance was evaluated by measuring the height of a vertical jump (VI).

The subjective feeling of exertion was rated according to a scale described by Borg (1974) (III-IV-V-VII).

Body temperatures have been measured by thermocouples except in study II where mercury rectal thermometers were used. Core temperature was continuously measured in the esophagus at the level of the heart. Muscle temperature in the vastus

lateralis muscle. Mean skin temperature was calculated from measurements made at 6-8 different skin locations (Saltin et al. 1968). The recording equipment and each sensor were calibrated at each individual trial.

The errors of the methods for the determination of variables are presented in Table 1.

### Subjects

In most of these studies only male subjects participated. They were all physically well trained and familiar with the laboratory procedures. Only in study II both male and female subjects took part. The subjects were informed about all aspects of the experiments before they consented to participate.

### Procedure

To avoid the influence of systematic errors and possible training effects, the order of the experimental situations was varied at random and a maximum of two experiments was performed per week with each subject.

### Statistics

Conventional statistical methods were used to calculate mean values, standard deviations and correlation coefficients. The t test was applied to analyze the significance of differences. The error of the method has been specified as the coefficient of variation (c.v.) of a single value and has been calculated from the following formula:

$$c.v. = \sqrt{\frac{1}{2} \frac{\sum (d - \bar{d})^2}{n}} \cdot \frac{1}{\bar{x}} \cdot 100$$

where  $d$  is the difference between double values,  $\bar{d}$  is the mean difference,  $n$  is the number of determinations and  $\bar{x}$  is the mean value.

### Comments

The vastus lateralis muscle was chosen for  $T_{ms}$  measurements and for obtaining muscle biopsies, since this muscle is highly active during the type of exercise performed in these experiments (Henriksson and Bonde-Pedersen 1974) and the risk of complications is minimal.

$T_m$  was measured immediately after exercise. The values presented are peak values obtained at a depth of 30-50 mm from the skin surface. In experiments where both  $T_m$  and muscle biopsies were obtained, the measurements were made as close to each other as possible (within 1 cm). In some of the present investigations attempts were made to quantify the effects of changes in body temperatures. In this context one faces the problem of evaluating the representativity of various temperature measurements. In the case of core temperature it was considered necessary to find a site of measurement which reflects the temperature both of the CNS and of the heart. This is essential when trying to relate both thermoregulatory and circulatory responses to internal temperature. The choice of esophageal temperature as the measure of core temperature in the present studies was dictated by two main considerations: 1)  $T_{es}$  responds faster than rectal temperature during the temperature transients of the exercise which might be important during heavy exercise of 3-8 min duration. Thus, rectal temperature will underestimate heart and brain temperature if these are increasing. 2) Also tympanic temperature has a fast response, but the measurements are usually rather unpleasant during heavy exercise, since the sensor tends to irritate the tympanic membranes as a result of the large ventilation and the body movements.

A teflon esophageal probe is usually tolerated very well by the subjects both at rest and during exercise, irrespective of the latter's severity.

A more difficult problem is to judge the representativity of a  $T_m$  measurement. The present investigations used the peak  $T_m$  values obtained at 30-50 mm from the skin surface. The primary reason was that it is easier to obtain reproducible values with this procedure compared to temperature measurements at a fixed depth. Another reason was that in an exercising muscle the highest value is likely to occur in the muscle's most active parts, and these areas might be of special importance with regard to physical performance. On the other hand, other parts of the muscle evidently contribute to total tension development. Hence it is not really possible to judge whether it is more relevant to relate the various changes to peak  $T_m$  or to average  $T_m$ . In the latter case one has to keep in mind that during cooling the

temperature gradients in the muscle are higher (Pugh 1967) Thus a  $T_m$  measurement at a point with above-average  $T_m$  will overestimate the temperature effect on various variables. Another problem is the above mentioned temperature transients which makes it more difficult to relate different variables to temperature. In order to minimize these problems temperature and other recordings were made as close to each other as possible (30 s). However the measurement of the total oxygen uptake (and oxygen deficit) is the exception from this since  $T_m$  was only measured before and after the exercise.

Table 1 Error of the method for some variables measured under different conditions expressed as the coefficient of variation

Esophageal temperature	rest (15 min)	separate days	0.3 %
	after 5 min of maximal exercise	separate days	0.1 %
Muscle temperature	rest (15 min)	separate days	1.4 %
	after maximal exercise	separate days	0.5 %
<u>Peak torque</u>			
0° 0/s	same day		4.5 %
90° 0/s			4.9 %
180° 0/s			6.7 %
0° 6/s	different days	highest value of 2 trials	6.1 %
90° 0/s	different days	highest value of 2 trials	6.5 %
180° 0/s	different days	highest value of 2 trials	7.0 %
<u>Vertical jump</u>			
	same day		3.5 %
	different days	highest value of 2 trials	1.5 %
<u>Maximal bicycling <sup>x)</sup></u>			
2.5 3s	separate days		2.4 %
10 15s			2.2 %
ATP CP G-6 P LA concentrations	see Essén 1978		
<u>Oxygen uptake</u>			
	submaximal exercise		1.5 %
	maximal exercise		2.1 %

x) speed

## RESULTS AND DISCUSSIONS

This section is divided into five parts

Rectal temperature after long distance swimming (II)

Aerobic power (I III IV V VII)

Anaerobic power (VI VII)

Maximal muscular strength (VI)

Physical performance (I II III IV V VI VII)

Rectal temperature after long distance swimming

The subjects were 8 females and 41 males of various ages (13-63 years) and training status (200-70 000 m of swimming per week). Only 15 of them were competitive swimmers. The time needed to finish the 3.2 km race ranged from 37 to 150 min (mean 72 min). Mean  $T_{re}$  before and after the race was  $37.7^{\circ}\text{C}$  ( $n=34$ ) and  $36.4^{\circ}\text{C}$  ( $n=49$ ) respectively. The comparatively high  $T_{re}$  at the start was due to warm up activities e.g. jogging, calisthenics.

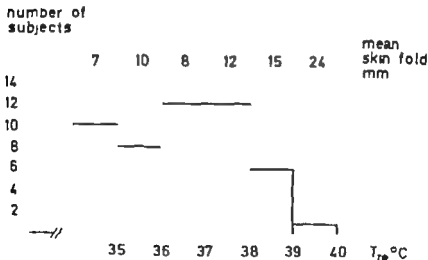


Fig. 1 The number of subjects who attained each level of rectal temperature ( $T_{re}$ ) at the end of 3.2 km swimming race (5 of the participants did not finish). The numbers in the upper part of the figure denote average mean skin fold value of the subjects in each of the  $T_{re}$  intervals. The water temperature was  $19^{\circ}\text{C}$  (II).

Despite this increased heat content of the body only 19 of the subjects had a  $T_{re}$  above  $37^{\circ}\text{C}$  at the finish thus the majority of the subjects had more or less subnormal  $T_{re}$  (Fig 1) and 10 of them reached a  $T_{re}$  below  $35^{\circ}\text{C}$ . Furthermore 5 of the participants were unable to complete the race and had to be helped out of the water. Their symptoms included shivering and cramps and in some cases dizziness. In contrast some subjects increased their  $T_{re}$  during the race.

The difference in  $T_{re}$  response was related both to skinfold thickness (cf Pugh & Edholm 1955 Keatinge 1969 Hadel et al 1974 Holmér & Bergh 1974a Boutelier et al 1977 Golden et al 1979) and to exposure of time the former seemed to be the more important one.

Evidently exposure of this type can produce subnormal body temperatures in most subjects.

### Aerobic power

Rest and submaximal exercise At  $T_{es}$  lower than  $36^{\circ}\text{C}$  oxygen uptake was increased above normal levels (I III IV)

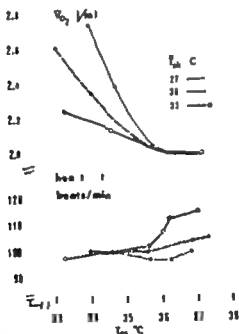


Fig 2 Oxygen uptake and heart rate on subject during submaximal exercise (150 W) at different core ( $T_{es}$ ) and mean skin temperature ( $T_k$ ) (From Bergh & Ekblom 1978)

The magnitude of this increase was inversely related both to  $T_{sk}$  (Fig 2) and to  $T_{es}$  (I). There are also data indicating an inverse relationship between the magnitude of the extra oxygen uptake and the rate of work (Fig 3). However the latter finding was not consistent.

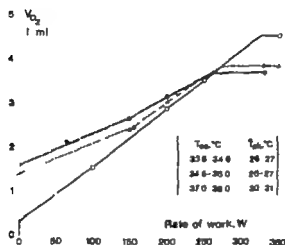


Fig 3 Oxygen uptake as function of the rate of work at normal and lowered body temperature. Note the levelling off at high rates of work (Average value = 3).

The extra oxygen uptake can be a result of shivering non-shivering thermogenesis or lowered mechanical efficiency during exercise. The major part of this extra energy yield is most likely an effect of shivering at least at rest where no work is performed and there is little evidence that non shivering thermogenesis contributes substantially to an increase in metabolic rate during acute cold exposure. There are reasons to believe that temperature can influence mechanical efficiency (e.g. by affecting nerve conductivity shivering interfering with coordination). However the present data do not support such an assumption.  $T_{es}$  could be changed considerably (from 37 to 35  $^{\circ}C$ ) without any substantial effect on the metabolic rate (Fig 2).



As mentioned earlier the magnitude of extra metabolic rate at subnormal  $T_{es}$  was affected by both  $T_{es}$  and  $T_{sk}$  indicating that shivering can be elicited by central as well as by peripheral cold stimuli. The latter seems to be more important (Benzinger et al 1963 Nielsen 1976). Another factor that might influence the magnitude of the extra metabolic rate is the rate at which work is performed. There are studies which indicate that the magnitude of the extra oxygen uptake is independent of the rate of work (Madel et al 1974 Nielsen 1976 1) but other data suggest an inverse relationship (Craig & Dvorak 1968 Hong & Madel 1979 and Fig 3).

The discrepancy between these results is partly of methodological origin. Nielsen studied a rather narrow range of metabolic rates which did not induce any substantial differences in the extra metabolic rate. As Madel et al (1974) did not examine the different levels of work intensities at equal body temperatures it is not possible to tell whether they induced any differences. It is however reasonable to believe that work intensity could influence the magnitude of shivering at least at higher rates of shivering involving a relatively large muscle mass. Hence as the rate of work increases more and more of the shivering muscle mass becomes involved in exercise and the magnitude of shivering would then decrease provided other muscles do not compensate by increasing their shivering activity. The purpose of shivering is to generate heat. Teleologically an increased rate of work (increased heat production) would diminish the need for shivering. A decrease in the magnitude of shivering would be a logical consequence even for muscles which are not voluntarily activated. In fact Hong & Madel (1979) found that shivering activity in the sternocleidomastoideus muscle of the neck was suppressed during bicycling and more so the heavier the work on the ergometer.

The cold induced increase in oxygen uptake was usually not accompanied by an elevation of heart rate which was unchanged or even reduced at rest (IV) and at a given rate of work (I-III) (cf Fig 2). This indicates that there must have been a change in either cardiac output stroke volume and/or arterio-venous  $O_2$  difference. McArdle et al (1976) reported that  $\dot{Q}$  and a  $VO_2$  difference were unchanged while SV at a given oxygen uptake was

higher during exercise in 18 °C water compared to 33 °C water. Thus at a given rate of work  $\dot{Q}$  and a  $\dot{V}O_2$  difference must have been higher in 18 °C. Their data are unfortunately not possible to relate to core temperature.

The reduced HR at a given oxygen uptake cannot be explained by a diminished sympathetic activity for the plasma norepinephrine concentration was higher at low body temperatures (IV Fig. 4).

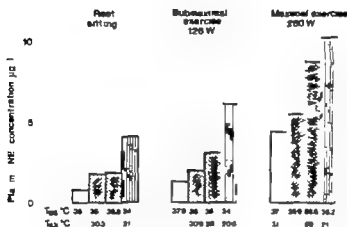


Fig. 4 Plasma norepinephrine (NE) concentration during submaximal and maximal exercise in different combinations of esophageal ( $T_{es}$ ) and mean skin temperature ( $T_{sk}$ ). Note that both low  $T_{es}$  and low  $T_{sk}$  can elevate the NE-concentration and that the effect seems to be additive (Average value  $n = 2$ ; IV).

However, the possibility still exists that  $\beta$ -receptor activity is reduced, which is supported by the fact that  $\beta$ -receptor blockade did not further reduce HR during exercise in hypothermia (III) in contrast to the reaction at normal body temperature (Ekblom et al. 1972). It is also possible that peripheral vasoconstriction enhances venous return and thereby increases SV. Furthermore, vasoconstriction can induce an elevated aBP and thereby elicit an increased activity in the pressor receptors which might depress HR in order to normalize aBP. At rest (sitting) aBP was higher at subnormal  $T_{es}$  and/or  $T_{sk}$  while during submaximal exercise aBP was the same or lower (IV). Furthermore

during submaximal exercise aBP was lowered in relation to oxygen uptake and HR at low  $T_{es}$  plus normal  $T_{sk}$  but not at normal  $T_{es}$  plus low  $T_{sk}$ . These findings may indicate that the observed depression of HR is due to a combined effect of a pressor receptor induced HR decrement and a reduced  $\beta$  receptor activity (IV).

One important implication of this HR response to lowered body temperatures is that predictions of physical work capacity on the basis of HR measurements during submaximal exercise will be misleading. Thus measures such as PWC<sub>170</sub>, watt pulse and oxygen pulse will lead to an overestimation of physical work capacity. If the estimation is based on measurements of HR at a given oxygen uptake the overestimation will be even greater than if the calculation is made from HR and rate of work, since oxygen uptake at a given rate of work is increased at subnormal  $T_{es}$  without a concomitant rise in HR.

Pulmonary ventilation at rest and during submaximal exercise increased with decreasing  $T_{es}$  and  $T_{sk}$  while the ventilatory equivalent was elevated only when a low  $T_{es}$  was combined with a low  $T_{sk}$  (unpublished results). This indicates that a cold skin constitutes an additional drive on the ventilatory center superimposed on the drive from the metabolic demand.

**Exhaustive exercise.** Peak oxygen uptake was positively related to  $T_{es}$  ( $r = 0.88$ ) and  $T_m$  ( $r = 0.91$ ) (V, Fig. 5). The rate of these changes was approximately  $5-6\% \text{ } ^\circ\text{C}^{-1}$ . Similar results have been obtained during swimming (Holmér & Bergh 1974b). No subject was able to attain his control maximal oxygen uptake at  $T_{es}$  and  $T_m$  lower than  $37.5$  and  $38.0 \text{ } ^\circ\text{C}$  respectively (V, Fig. 6). This response to variation in  $T_{es}$  and  $T_m$  was unaltered by a moderate change in  $T_{sk}$  (cf Fig. 6 & 7). However, it should be kept in mind that  $T_{sk}$  will sooner or later affect  $T_{es}$  and  $T_m$  (cf swimming in cold water) and thereby cause considerable indirect effects on different variables (even if their dependence of  $T_{es}$  and  $T_m$  is not modified directly).

Lower  $T_{es}$  and  $T_m$  did not affect oxygen uptake response during the first two minutes of exercise (V, VII). Thereafter oxygen uptake was related to  $T_{es}$  and  $T_m$ , i.e. at normal temperatures oxygen uptake continued to increase significantly while

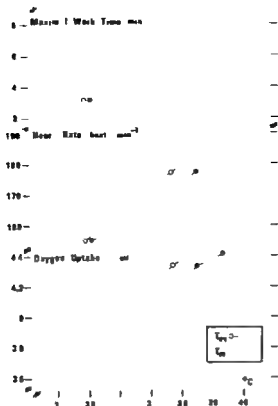


Fig. 5. A graph showing the relationship between time to exhaustion, peak heart rate and peak oxygen uptake in relation to esophageal ( $T_{es}$ ) and muscle ( $T_m$ ) temperature (°C).

it levelled off at subnormal  $T_{es}$  and  $T_m$  in spite of a constant rate of work.

Peak HR was related to  $T_{es}$  and  $T_m$  in the same manner as peak oxygen uptake (V Fig. 5 & 7) which is in accordance with data obtained during swimming (Nadel et al. 1974; Holmér & Bergh 1974a). The difference between the highest HR attained by each subject (regardless of temperature) and the HR peak at respective  $T_{es}$  and  $T_m$  was correlated both to  $T_{es}$  and to  $T_m$  ( $r = 0.97$  and  $0.95$  respectively) and the effect of temperature on peak HR amounted to 8 beats  $\text{min}^{-1} \text{ } ^\circ\text{C}^{-1}$ .

The data on HR during the first min of exercise are contradictory in two studies. HR was unaltered by  $T_{es}$  and  $T_m$  (V) and parasympathetic blockade. This discrepancy might originate from the fact that lower relative work rates were performed in study III and V than in study VII.

during submaximal exercise ABP was lowered in relation to oxygen uptake and HR at low  $T_{es}$  plus normal  $T_{sk}$  but not at normal  $T_{es}$  plus low  $T_{sk}$ . These findings may indicate that the observed depression of HR is due to a combined effect of a pressor-receptor induced HR decrement and a reduced  $\beta$  receptor activity (IV).

One important implication of this HR response to lowered body temperatures is that predictions of physical work capacity on the basis of HR measurements during submaximal exercise will be misleading. Thus measures such as  $\dot{V}O_{2max}$  watt pulse and oxygen pulse will lead to an overestimation of physical work capacity. If the estimation is based on measurements of HR at a given oxygen uptake the overestimation will be even greater than if the calculation is made from HR and rate of work since oxygen uptake at a given rate of work is increased at subnormal  $T_{es}$  without a concomitant rise in HR.

Pulmonary ventilation at rest and during submaximal exercise increased with decreasing  $T_{es}$  and  $T_{sk}$ , while the ventilatory equivalent was elevated only when a low  $T_{es}$  was combined with a low  $T_{sk}$  (unpublished results). This indicates that a cold skin constitutes an additional drive on the ventilatory center superimposed on the drive from the metabolic demand.

**Exhaustive exercise** Peak oxygen uptake was positively related to  $T_{es}$  ( $r = 0.88$ ) and  $T_{m}$  ( $r = 0.91$ ) (V, Fig. 5). The rate of these changes was approximately  $5-6\% ^\circ C^{-1}$ . Similar results have been obtained during swimming (Holmér & Bergh 1974b). No subject was able to attain his control maximal oxygen uptake at  $T_{es}$  and  $T_{m}$  lower than  $37.5$  and  $38.0 ^\circ C$  respectively (V, Fig. 6). This response to variation in  $T_{es}$  and  $T_{m}$  was unaltered by a moderate change in  $T_{sk}$  (cf Fig. 6 & 7). However, it should be kept in mind that  $T_{sk}$  will sooner or later affect  $T_{es}$  and  $T_{m}$  (cf swimming in cold water) and thereby cause considerable indirect effects on different variables (even if their dependence of  $T_{es}$  and  $T_{m}$  is not modified directly).

Lower  $T_{es}$  and  $T_{m}$  did not affect oxygen uptake response during the first two minutes of exercise (V, VII). Thereafter oxygen uptake was related to  $T_{es}$  and  $T_{m}$ . At normal temperatures oxygen uptake continued to increase significantly while

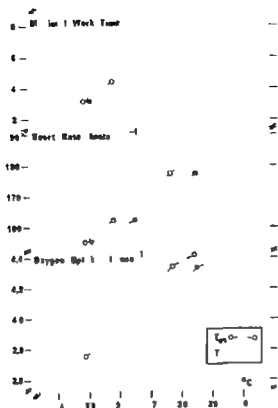


Fig 5 Average value (  $\pm$  SE ) of time to exhaustion peak heart rate and peak oxygen uptake in relation to oesophageal ( $T_{es}$ ) and vastus lateralis temperature ( $T_m$ ) (°C)

it levelled off at subnormal  $T_{es}$  and  $T_m$  in spite of a constant rate of work

Peak HR was related to  $T_{es}$  and  $T_m$  in the same manner as peak oxygen uptake (V Fig 5 & 7) which is in accordance with data obtained during swimming (Nadel et al 1974 Holmér & Bergh 1974a). The difference between the highest HR attained by each subject (regardless of temperature) and the HR peak at respective  $T_{es}$  and  $T_m$  was correlated both to  $T_{es}$  and to  $T_m$  ( $r = 0.97$  and  $0.95$  respectively) and the effect of temperature on peak HR amounted to 8 beats  $\text{min}^{-1} \text{ } ^\circ\text{C}^{-1}$ .

The data on HR during the first min of exercise are contradictory in two studies. HR was unaltered by  $T_{es}$  and  $T_m$  (V) and parasympathetic blockade. This discrepancy might originate from the fact that lower relative work rates were performed in study III and V than in study VII.

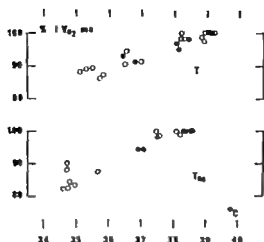


Fig. 6 Peak oxygen uptake at the end of exhaustive exercise for 3-8 min in per cent of as high as possible maximal oxygen uptake obtained at 2 levels of mean skin temperature ( $T_{sk}$ ) in relation to difference in oxygen uptake ( $T_{sk}$  lower panel) and vastus lateralis ( $T_{sk}$  upper panel) temperature as filled circles  $T_{sk} 27^{\circ}\text{C}$  pen-  
cils  $T_{sk} 31^{\circ}\text{C}$  (Data on 8 subjects; V)

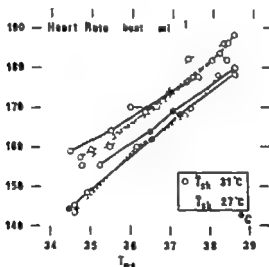


Fig. 7 Heart rate at the end of exhaustive exercise at 2 levels of mean skin temperature ( $T_{sk}$ ) (8 V)

What effect of lowered  $T_{es}$  and/or  $T_{in}$  can cause the decrement in peak oxygen uptake? The shorter work time may of course prevent oxygen uptake from accelerating to its maximum potential. However, at the first two min of exercise ( $V$  and  $VII$ ) oxygen

uptake was the same irrespective of temperature but it levelled off at a lower value at subnormal  $T_{es}$  and  $T_m$  compared to normal or elevated body temperatures (V). Neither can a relative hypoventilation explain the reduction in peak oxygen uptake since the pulmonary ventilation was the same and the ventilatory equivalent was higher at low body temperatures (V). Oxygen diffusion rate is of course related to temperature but hardly to an extent that would induce a measureable change in peak oxygen uptake within the temperature range used in the present study. The most conspicuous finding in relation to the decrement in peak oxygen uptake is the lowered peak HR especially since the change in peak HR was well related to the change in peak oxygen uptake ( $r = 0.78$ ).

Temperature may affect HR in many ways e.g. 1) by a modification of the activity in the autonomous nervous system 2) by causing a reflex activity from the pressor receptors 3) by affecting the heart muscle (its ability to produce tension). Since atropin did not alter this HR response (III) an increased para sympathetic activity is not a likely explanation for the reduction in peak HR. Neither can a decreased sympathetic activity serve as a plausible explanation for lowered body temperatures were accompanied by greatly increased plasma NE concentrations (IV). However  $\beta$  receptor activity might still be decreased by lowered tissue temperature. A reflex activity from pressor-receptors as a mediator of a HR reduction is less probable considering that aBP during maximal exercise was lower at lowered  $T_{es}$  (IV).

The present data accordingly indicate that the decrement in peak HR can result from a direct effect of temperature on the heart muscle. Thus the speed of contraction of any muscle is dependent on the load (force) against which it is contracting. An increase in the resisting force will inevitably result in a decrease in the maximal speed of contraction. Thus for a given stroke volume and blood pressure (afterload) there is a maximal speed of contraction which cannot be exceeded. A lowered tissue temperature will induce a leftward shift in the force velocity relationship (at a given force the maximal velocity is decreased). Thus peak HR should theoretically decrease if the temperature of the heart muscle is lowered. A direct effect of



temperature on the heart muscle is indicated by the facts that at low tissue temperature 1) there was an increase in the time necessary to reach peak tension and a reduction in the tension increment per unit of time in isolated heart muscle preparations (Ekblom & Bing 1979) 2) the effect of temperature on peak HR was of the same order of magnitude as that in maximum muscle strength observed in skeletal muscles (VI) Two main possibilities are available for explaining the reduction in peak HR 1) decreased  $\beta$  receptor activity 2) reduced force development The observed reduction in aBP may be a consequence of a reduced cardiac performance

### Anaerobic power

The immediate source of energy for muscle contraction is ATP The normal ATP stores are however consumed by a few maximal contractions A prolongation of muscular exercise necessitates resynthesis of ATP Anaerobically ATP can be produced via a breakdown of CP and by glycogenolysis and glycolysis The stores of CP can be depleted in less than 10 s (Karlsson 1971) while the stores of glycogen are much larger and are not normally emptied by exhaustive exercise of 2-3 min duration The maximal rate at which energy is delivered through these various processes differs For these reasons it is necessary to study power output during exercise of varying duration in order to investigate the effect of temperature on the different candidates yielding energy It should be emphasized that these experiments were carried out after local cooling of the legs Thus there was little or no shivering

For a single muscle contraction (knee extension), peak power output was positively related to temperature The magnitude of change was  $4.6^{\circ}\text{C}^{-1}$  (VI) Average power output during the first revolutions of maximal bicycling and the height of a maximal vertical jump (VI) were affected qualitatively and quantitatively in the same way as peak power output during knee extensions These findings cannot be explained by differences in the quantities of available ATP since its concentration was unaffected by  $T_{\text{in}}$  (Edwards et al 1972 VII) Thus it is more likely that tissue temperature affects the rate of ATP breakdown and/or the transformation from chemical energy to mechanical work

At a maximal effort lasting 5 to 10 s e.g. maximal bicycling CP breakdown is the main anaerobic source for ATP resynthesis and thus energy yield. In this type of exercise power output changed as described for peak power and during knee extensions (VI). This must mean that ATP production by CP-breakdown was not more affected by lowering of  $T_m$  than was ATP breakdown and/or that the latter step is the rate limiter.

When exercise is performed at a rate which leads to exhaustion in 1 to 3 min the major part of the anaerobic energy is derived through glycogenolysis (Karlsson & Saltin 1970). In this type of exercise (VII) time to exhaustion was shorter at subnormal  $T_m$ . Average values were 1.8 min at  $T_m$  35.0 °C and 2.5 min at  $T_m$  37.1 °C. Oxygen deficit and blood LA-concentration (both at exhaustion and the peak value) were lower while muscle LA-concentration was somewhat higher at low  $T_m$  (Fig. 8). The

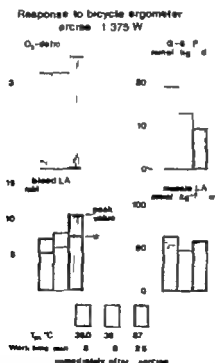


Fig. 8. Oxygen deficit and the change ( $\Delta$ ) of glucose-6-phosphat blood and muscle concentrations of lactate (LA) during bicycling exercise (375 W) at different rectal temperatures ( $T_m$ ) and work time. Note that the subject was exhausted at 1.8 min at low  $T_m$  while the same work rate was sustained for 2.5 min at normal  $T_m$  (n = 4; VII).

temperature on the heart muscle is indicated by the facts that at low tissue temperature 1) there was an increase in the time necessary to reach peak tension and a reduction in the tension increment per unit of time in isolated heart muscle preparations (Ekblom & Bing 1979) 2) the effect of temperature on peak HR was of the same order of magnitude as that in maximum muscle strength observed in skeletal muscles (VI) Two main possibilities are available for explaining the reduction in peak HR 1) decreased  $\beta$  receptor activity 2) reduced force development The observed reduction in aBP may be a consequence of a reduced cardiac performance

### Anaerobic power

The immediate source of energy for muscle contraction is ATP The normal ATP stores are however consumed by a few maximal contractions A prolongation of muscular exercise necessitates resynthesis of ATP Anaerobically ATP can be produced via a breakdown of CP and by glycogenolysis and glycolysis The stores of CP can be depleted in less than 10 s (Karlsson 1971) while the stores of glycogen are much larger and are not normally emptied by exhaustive exercise of 2-3 min duration The maximal rate at which energy is delivered through these various processes differs For these reasons it is necessary to study power output during exercise of varying duration in order to investigate the effect of temperature on the different candidates yielding energy It should be emphasized that these experiments were carried out after local cooling of the legs Thus there was little or no shivering

For a single muscle contraction (knee extension), peak power output was positively related to temperature The magnitude of change was  $4.6\% \text{ } ^\circ\text{C}^{-1}$  (VI) Average power output during the first revolutions of maximal bicycling and the height of a maximal vertical jump (VI) were affected qualitatively and quantitatively in the same way as peak power output during knee extensions These findings cannot be explained by differences in the quantities of available ATP since its concentration was unaffected by  $T_m$  (Edwards et al 1972 VII) Thus it is more likely that tissue temperature affects the rate of ATP breakdown and/or the transformation from chemical energy to mechanical work

rate of LA removal as indicated by the lower rate of blood LA increase during exercise at low  $T_{re}$  (this can be induced by a reduced flow lower diffusion rate and/or decreased LDH-activity) But it is also conceivable that the  $O_2$ -deficit calculated from oxygen uptake measured over the lungs does not mirror oxygen uptake over the working muscles in the same way at different body temperature levels. An  $O_2$  deficit in these muscles can be underestimated if there is an elevated oxygen uptake in other tissues e.g. as a result of shivering. This is probably not the case in the present experiments since oxygen uptake at rest immediately before the start of exercise was the same in all experiments performed after local cooling of the legs.  $\beta$ -receptor blockade can decrease LA removal from an exercising muscle (Juhlin-Dannfeldt & Åström 1979) i.e. a similar event as noted in the low  $T_{re}$  experiments. As discussed on p. 19 a reduced  $T_{es}$  may suppress the  $\beta$ -receptor activity.

#### Muscular strength

Maximal isometric strength was little affected by  $T_{re}$  ( $2 \pm 0.1^\circ C^{-1}$ ) whereas  $T_{re}$  did have a considerable effect on maximal dynamic strength at all studied speeds of contraction ( $4.5 \pm 0.1^\circ C^{-1}$ ). Thus the force-velocity curve was shifted to the left (Fig. 9). The present results on maximal muscle strength are in agreement with previous studies in humans (Asmussen et al. 1976; Binkhorst et al. 1977. For refs. see Fallis 1972). In contrast Asmussen et al. (1976) demonstrated that during eccentric contraction the capability to store tension (produced e.g. by a downward jump) is enhanced by a decrement in  $T_{re}$ . This is further supported by the finding that the ability to perform eccentric bicycle exercise was lowered at very high  $T_{re}$  (Nadel et al. 1972). Furthermore endurance in isometric exercise is reported to be enhanced at subnormal  $T_{re}$ . Thus the influence of temperature on muscle strength is different in different types of exercise. The cause of this phenomenon is still unknown. Asmussen et al. (1976) suggested that temperature influences the rate of breaking and formation of the cross bridges between the actin and the myosin filaments. The above-mentioned results fit this hypothesis very well since such a mechanism would involve a resistance to any change in muscle length. Thus in eccentric and in isometric

endurance exercise the resistance will enhance external force development and vice versa in concentric exercise. Maximal isometric strength should consequently be unaffected by temperature. However, an isometric contraction usually has an initial shortening phase in regard to the muscle filament. A small negative influence on force development would then be expected and has in fact been reported (Åstrand et al 1976 VI).

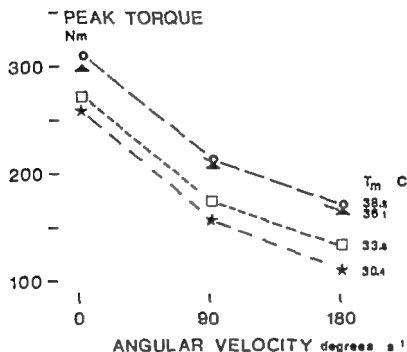


Fig 9 Peak torque as function of angular velocity of the knee joint during extension (relationship of velocity and torque) at different levels of  $T_m$  (Åstrand et al 1976 VI)

It is also interesting to note that the temperature effect on power was of the same order of magnitude over a wide range of speed of movement (measured during bicycling VI).

### Physical performance

Physical performance is in a broad sense a function of energy output, neuromuscular function and psychological factors. The present series of experiments concentrated primarily on the

first of these factors. However some information about the other two has also been obtained.

Performance was found to be decreased at subnormal temperatures in exercises consisting of one up to several hundred contractions. This is not surprising since factors affecting the energy yield (both aerobic and anaerobic) were negatively influenced by a lowered tissue temperature. In exercise lasting longer than 2 min the major part of the energy demand is covered by aerobic processes. Therefore the observed decrement in peak oxygen uptake will have an adverse effect on performance in such types of exercise. If performance is expressed as  $W_{\max 6}$  (i.e. a prediction of the rate of work that can be performed during 6 min based on maximal work time for any exhaustive rate of work; see Tornvall 1963) the change corresponded to approximately  $8\% \text{ } ^\circ\text{C}^{-1}$  for exercises lasting 3-8 min.

In exhaustive exercise for less than 2 min the change in  $W_{\max 6}$  was  $5-6\% \text{ } ^\circ\text{C}^{-1}$  which is of the same order of magnitude as the difference in maximal power output in 1-20 contractions. The obvious explanation for the greater effect of body temperature on performance in the former type of exercise is that there is a change in both aerobic and anaerobic energy yield in contrast to short time exercise where oxygen uptake at a given work time was independent of temperature.

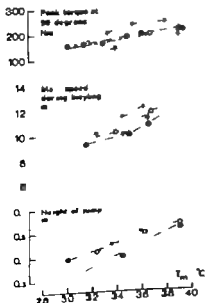


Fig. 10. Individual effect of peak torque during knee extension, maximal speed of the fly-wheel during bicycling of 10-15 min and height of vertical jump at different values of  $T_m$  (4; VI).

Performance in the sprinting and jumping tests was positively related to  $T_{sk}$  (Fig 10) The decrements were proportional to the changes in peak torque (maximal dynamic strength) thus indicating that factors such as coordination were little affected One must however bear in mind that the jumping and sprinting tests consisted of rather uncomplicated movements (stationary bicycling and vertical jumping) which require fairly little skill Furthermore the experiments were carried out at ambient temperatures of 22-24 °C after local cooling of the legs which induced quite small effects on  $T_{os}$  and  $T_{sk}$  thus resulting in minimal shivering Severe shivering might interfere with an optimal coordination and thereby cause a further decrement in performance without a concomitant decrement in maximal muscle strength (unpublished results) Very low  $T_{sk}$  will also inhibit sensory impulses from skin receptors for pressure and touch which might result in very severe disturbance in the motor function

The increased metabolic rate for a given task will of course lead to a faster depletion of energy stores of which glycogen and glucose are the more critical for prolonged exercise (especially when this leads to hypoglycemia which may affect thermoregulation) Alcohol consumption in combination with reduced liver glycogen stores can elicit hypoglycemia and thus indirectly cause a fall in body temperature in individuals exposed to a cold environment (Haight & Keatinge 1973)

The energy cost (oxygen uptake) at a given rate of work is higher and the peak oxygen uptake is lower at lower body temperature and hence a reduced energy pool is available for physical work This indicates a greater relative strain on the individual It is however of interest to note that the subjects do not seem to have perceived any augmented feeling of fatigue they did not rate their perceived exertion (RPE) any higher at a given rate of work given oxygen uptake or given HR at lower body temperatures (Fig 11) The tendency is rather the opposite thus indicating that the subjects are unaware of the increase in the relative rate of work and the reduced potential for heavy exercise

During swimming in cold water however RPE was higher at given values for submaximal speed and HR (Fig 11) than in warm

water. This discrepancy between the different types of exercise can result from a more pronounced cooling of the arms in combination with their greater relative involvement during swimming compared to bicycling. It should be emphasized that tissue temperature mostly decreases during swimming in cold water while during bicycling at room temperature the opposite occurs. Thus in bicycling the feeling of cold stress decreases gradually while it increases during swimming. These observations indicate that the normal perception of exertion in relation to the rate of work is considerably altered by changes in temperature stimuli a fact that might be of importance in many situations.

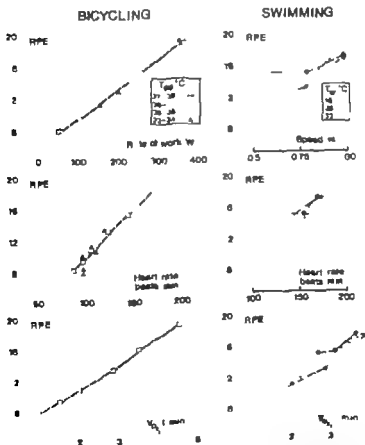


Fig 11 Related perceived exertion (RPE) in relation to exercise intensity (upper panel) heart rate and oxygen uptake (lower panel) at different ambient temperatures (bicycling) and water temperature ( $T_w$ ) (swimming).



Performance in the sprinting and jumping tests was positively related to  $T_{re}$  (Fig 10) The decrements were proportional to the changes in peak torque (maximal dynamic strength) thus indicating that factors such as coordination were little affected One must however bear in mind that the jumping and sprinting tests consisted of rather uncomplicated movements (stationary bicycling and vertical jumping) which require fairly little skill Furthermore the experiments were carried out at ambient temperatures of 22-24 °C after local cooling of the legs which induced quite small effects on  $T_{es}$  and  $T_{sk}$  thus resulting in minimal shivering Severe shivering might interfere with an optimal coordination and thereby cause a further decrement in performance without a concomitant decrement in maximal muscle strength (unpublished results) Very low  $T_{sk}$  will also inhibit sensory impulses from skin receptors for pressure and touch which might result in very severe disturbance in the motor function

The increased metabolic rate for a given task will of course lead to a faster depletion of energy stores of which glycogen and glucose are the more critical for prolonged exercise (especially when this leads to hypoglycemia which may affect thermoregulation) Alcohol consumption in combination with reduced liver glycogen stores can elicit hypoglycemia and thus indirectly cause a fall in body temperature in individuals exposed to a cold environment (Haight & Keatinge 1973)

The energy cost (oxygen uptake) at a given rate of work is higher and the peak oxygen uptake is lower at lower body temperature and hence a reduced energy pool is available for physical work This indicates a greater relative strain on the individual It is however of interest to note that the subjects do not seem to have perceived any augmented feeling of fatigue they did not rate their perceived exertion (RPE) any higher at a given rate of work given oxygen uptake or given HR at lower body temperatures (Fig 11) The tendency is rather the opposite thus indicating that the subjects are unaware of the increase in the relative rate of work and the reduced potential for heavy exercise

During swimming in cold water however RPE was higher at given values for submaximal speed and HR (Fig 11) than in warm

## SUMMARY

Data are presented from studies analyzing the following

- The incidence and extent of body cooling during a 3.2 km swimming race in water at 19°C
- The effect of body temperatures on
  - aerobic power at rest and during submaximal and maximal exercise
  - anaerobic power at maximal efforts ranging from fractions of a second to 3 min
  - maximal muscle strength at different speeds of movement
  - physical performance evaluated in the following ways
    - Work time 1.5 - 8 min time to exhaustion during a standard maximal rate of work (bicycling) from which  $\dot{V}_{O_{2max}}$  was calculated
    - Work time 1 - 15 s the velocity (of the fly-wheel) attained at maximal bicycle ergometer exercise with a given resistance (torque)
    - Work time < 1 s height of a vertical jump

- 1 Swimming 3.2 km in water at 19°C produced a subnormal core temperature in the majority of the participants
- 2 At rest and during submaximal exercise lowered esophageal ( $T_{es}$ ) and mean skin ( $T_{sk}$ ) temperature induced an increase in oxygen uptake above normal levels. This increase was attributed to shivering which was inversely related to  $T_{sk}$ ,  $T_{es}$  and less consistently to the rate of work
- 3 Heart rate (HR) was lower at a given oxygen uptake at rest and during submaximal exercise at subnormal  $T_{es}$  and/or  $T_{sk}$
- 4 Peak aerobic power during exhaustive exercise of 3 - 8 min duration was positively related to  $T_{es}$  and  $T_m$  (muscle temperature) ( $r = 0.89$  and  $0.91$  respectively). These relations were unaltered by moderate changes in  $T_{sk}$

- 5 Peak HR was related to  $T_{es}$ ,  $T_m$  and  $T_{sk}$  in the same way as oxygen uptake. The correlation between changes in HR and changes in oxygen uptake was 0.78.
- 6 The calculated maximal anaerobic power was positively related to  $T_m$  in exercises lasting from 0.5 s to 3 min. The effect of temperature was  $4-6\% \text{ } ^\circ\text{C}^{-1}$ .
- 7 At work time < 2 min low  $T_m$  induced a faster rate of LA accumulation in the working muscle, probably attributable to a lower rate of LA removal (LA = lactic acid). At work time 3-8 min this was combined with a reduced aerobic power.
- 8 ATP and CP concentrations in the muscle showed no consistent relation to  $T_m$ , either at rest or immediately after exercise.
- 9 Maximal muscle strength was positively related to  $T_m$ . This effect of temperature amounted to  $2\% \text{ } ^\circ\text{C}^{-1}$  and  $4-6\% \text{ } ^\circ\text{C}^{-1}$  in isometric and dynamic exercise, respectively.
- 10 Physical performance was positively related to  $T_{es}$  and  $T_m$ . The temperature effect amounted to  $4-6\% \text{ } ^\circ\text{C}^{-1}$  in maximal exercise of less than 3 min duration, and  $8\% \text{ } ^\circ\text{C}^{-1}$  at 3-8 min exercise.

## ACKNOWLEDGEMENTS

I wish to express my gratitude to

Professor Per-Olof Astrand Head of the Department of Physiology III Karolinska Institute Stockholm for working facilities generous support and helpful discussions

Professor Erik Hohwul Christensen former Head of the Department for valuable advice in many respects

Associate Professor Björn Ekblom for stimulating discussions guidance never failing support and interest

Professor Bengt Saltin and fil lic Rune Hedman who introduced me to experimental work

My colleagues for pleasant and inspiring collaboration;

Dr Birgitta Essén and civil engineer Eva Blomstrand for helpful discussions and excellent technical assistance

Mrs Inger Hallbläck Mrs Liv Rydén Mrs Berit Sjöberg Mrs Ann-Chatrine Samuelsson and Mrs Anne-Britt Olrog for skilful technical assistance

Mr Eddy Karlsson Mr Harry Hagelin and Mr Georg Goertz for their untiring work in keeping the instruments functioning

Mrs Ulla Siltberg and Mrs Marie-Louise Eschicht for excellent secretarial help

All the subjects who volunteered for the studies

These studies have been supported by grants from the Research Council of the Swedish Sports Federation

## REFERENCES

- ASMUSSEN E BOMDE PETERSEN F & JØRGENSEN K 1976 Mechano-elastic properties of human muscles at different temperatures *Acta Physiol Scand* 96 83-93
- ASMUSSEN E & BØJE Ø 1945 Body temperature and capacity for work *Acta Physiol Scand* 10 1-22
- ASTRAND P-O & RODAHL K 1977 *Textbook of Work Physiology* McGraw-Hill New York
- BENZINGER T H KITZINGER & PRATT A W 1963 The human thermostat. In *Temperature its measurement and control in science and industry* (ed J D Hardy) vol 3 part 3 pp 637-665 Reinhold New York
- BERGH U & EKBLOM B 1978 Aerobic Power during exercise at varying body temperatures *Swimming Medicine IV* (eds B Ø Eriksson & B Furberg) pp 323-326 University Park Press Baltimore
- BERGH U KANSTRUP J L EKBLOM B 1976 Maximal oxygen uptake during exercise with various combinations of arm and leg work *J Appl Physiol* 41 191-196
- BERGSTROM J 1962 Muscle electrolytes in man *Scand J clin Lab Invest Suppl* 68
- BINKHORST R A MOOFD L & VISSERS A C A 1977 Temperature and force-velocity relationship of human muscles *J Appl Physiol Respirat Environ Exercise Physiol* 42 471-475
- BORG G A V & NOBLE B J 1974 Perceived Exertion. In *Exercise and Sport Sciences Reviews* vol 2 (ed Jack H Wilmore) Academic Press New York and London
- BOUTELIER C BOUGUES L & TIMBAL J 1977 Experimental study of convective heat transfer coefficient for the human body in water *J Appl Physiol Respirat Environ Exercise* 42 93-100
- BURTON A C & EDHOLM O G 1955 *Man in cold environment* Edward Arnold London

- HOLMÉR I & BERGH U 1974a Metabolic and thermal response to swimming in water at varying temperatures J Appl Physiol 37 702-705
- HOLMÉR I & BERGH U 1974b Hypothermia and physical work capacity of swimming man In Selected topics in environmental biology International symposium on exercise and sports physiology pp 163-167 Interprint publ New Delhi India
- HONG S I & MADEL E R 1979 Thermogenetic control during exercise in cold environment J Appl Physiol Respirat Environ Exercise Physiol 47 1084-1089
- HUGBERG P & LJUNGGREN B 1947 Uppvärmningens inverkan på löpprestationerna (The influence of warm-up on running performance) Svensk Idrott 40 668-671
- JUHLIN DAMMFELDT A & ÅSTRÖM H 1979 Influence of  $\beta$  receptor blockade on leg blood flow and lactate release in man Scand J clin Lab Invest 39 179-183
- KARLSSON J 1971 Lactate and phosphagen concentrations in working muscle of man Acta Physiol Scand Suppl 358
- KARLSSON J & SALTIN B 1970 Lactate ATP and CP in working muscles during exhaustive exercise in man J Appl Physiol 29 598-562
- KEATINGE W B 1969 Survival in cold water Blackwell Oxford & Edinburgh England
- LAKE C R, ZIEGHER H G & KOPKIN I J 1978 Use of plasma norepinephrine for evaluation of sympathetic neural function in man Life Sci 40 1315-1328
- LIND A R 1959 Muscle fatigue and recovery from fatigue induced by sustained contractions J Physiol 127-162 171
- MACLEAN D & ERNSTIE SMITH B 1977 Accidental hypothermia Blackwell Oxford & Edinburgh England
- MCARDLE W P, PAGE J R, LESMES G R & PECHAR G S 1976 Metabolic and cardiovascular adjustments to work in air and water at 18, 25, 33 °C J Appl Physiol 40 85-90
- MUJDO L 1947 The influence of body temperature on performances in swimming Acta Physiol Scand 12 102-109
- MADEL E R, BERGH U & SALTIN B 1972 Body temperatures during negative work exercise J Appl Physiol 33:553-558
- MADEL E R, HOLMÉR I, BERGH U, ÅSTRAND P-O & STOLWIJK J A J 1974 Energy & change of swimming man J Appl Physiol 36 465-471

- CRAIG A B Jr & DVORAK M 1968 Thermal regulation of man exercising during water immersion J Appl Physiol 25 28-35
- EDWARD R T H HARRIS R C HULTMAN E KAIJSER L KOH D & MORDENSTU L ■ 1972 Effect of temperature on muscle energy metabolism and endurance during successive isometric contractions sustained to fatigue of the quadriceps muscle in man J Physiol (Lond) 220 335-352
- EKBLOM ■ & BING O H L 1979 Effect of temperature change on the tolerance of isolated contracting cardiac muscle to hypoxia Cryobiology 16 161-165
- EKBLOM ■ GOLDBERG A KILBOM A & ASTRAND P O 1972 Effect of atropine and propranolol on the oxygen transport system during exercise in man Scand J clin Lab Invest 30 35-42
- ESSEN B 1978 Studies on the regulation of metabolism in human skeletal muscle using intermittent exercise as an experimental model Acta Physiol Scand Suppl 454
- FALLS H B 1972 Heat and cold applications In Ergogenic aids and muscular performance (ed W P Morgan) pp 119-158 Academic Press New York
- GLOSSARY COMMITTEE OF THE I U P S 1973 a Glossary on respiration and gas exchange J Appl Physiol 34 549-558
- GLOSSARY COMMITTEE OF THE I U P S 1973b Glossary of terms for thermal physiology J Appl Physiol 35 941-961
- GOLDEN F St C HAMPTON I F G HERVEY G R & KNIBBS A Y 1979 Shivering intensity in humans during immersion in cold water J Physiol 290 48P
- HAIGHT J S J & KEATINGE W R 1973 Failure of thermoregulation in the cold during hypoglycaemia induced by exercise and ethanol J Physiol 229 87-97
- HALLBACK I KARLSSON E & EKBLOM B 1978 Comparison between mass spectrometry and Haldane technique in analyzing O<sub>2</sub> and CO<sub>2</sub> concentrations in air gas mixture Scand J clin Lab Invest 38 285-288
- HENRIKSSON J ■ BONDE PETERSEN F 1974 Integrated electromyography of quadriceps femoris muscle at different exercise intensities J Appl Physiol 36 218-220
- HOLMER I 1972 Oxygen uptake during swimming in man J Appl Physiol 33 502-509

- NIELSEN B 1976 Metabolic reactions to changes in core and skin temperature in man *Acta Physiol Scand* 97 125-132
- PUGH L G C 1967 Cold stress and muscular exercise with special reference to accidental hypothermia *British Medical Journal* 2 333-337
- PUGH L G C & EDHOLM O G 1955 The physiology of channel swimmers *Lancet* II 761-768
- SALTIN B GAGGE A P & STOLWIJK J A J 1968 Muscle temperature during submaximal exercise in man *J Appl Physiol* 25 679-688
- STRÖM B 1949 The influence of anoxia on lactate utilization in man after prolonged muscular work *Acta Physiol Scand* 17 440-45
- TORRYALL G 1963 Assessment of physical capabilities *Acta Physiol Scand Suppl* 201









ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 479

SYMPOSIUM ON  
THE EFFECTS OF NICOTINE  
ON NERVOUS FUNCTIONS

Edited by  
THE MEDICAL ADVISORY BOARD OF  
THE SWEDISH TOBACCO COMPANY  
Stockholm Sweden



**Acta Physiologica Scandinavica**  
**Supplementum 479**

# **Symposium on the Effects of Nicotine on Nervous Functions**

Edited by  
The Medical Advisory Board of  
the Swedish Tobacco Company  
Stockholm Sweden

**Stockholm 1980**



## Contents

- NORDBERG A & BUNDWALL, A Assessment of cholinergic neuronal activity in the brain 7
- PILOTTI A Biosynthesis and membran metabolism of nicotine 13
- NORDBERG A, & LARSSON C Studies of muscarinic and nicotinic binding sites in brain 19
- JONSSON G & HALLMAN H Effects of neonatal nicotine administration on the postnatal development of central noradrenergic neurons 25
- ANDERSSON K, FUXE, K ENEROTH P GUSTAFSSON J Å & AGNA TI L F Mecamylamine induced blockade of nicotine induced inhibition of gonadotrophin and TSH secretion and of nicotine induced increases of catecholamine turnover in the rat hypothalamus 27
- SVENSSON T H & ENGBERG G Effect of nicotine on single cell activity in the noradrenergic nucleus locus coeruleus 31
- HÄGGENDAL, J & HENNING M Effect of chronically administered nicotine on axonal transport of dopamine- $\beta$ -hydroxylase in peripheral adrenergic neurons and on blood pressure and heart rate in the rat 35
- OWMAN CH AUBINEAU P EDVINSSON L & SERCOMBE, R Cholinergic inhibition of sympathetic vasoconstrictor tone in the cerebrovascular bed mediated by nicotine-type receptors 39
- BRUNDIN T Effects of tobacco smoking on the blood temperature during exercise 43
- ALSTER, P & WENNMALM Å Effect of nicotine on the formation of prostaglandin in the rabbit kidney 49
- SCHALLING D & WALLER, D Psychological effects of tobacco smoking 53





## Introduction

The Medical Advisory Board of the Swedish Tobacco Company was formed in 1957 in consultation with the National Board of Health. The main task of the Board is to stimulate medical, biological and chemical research into the effects of the use of tobacco and to advise the Company in sponsoring such activity in Sweden.

Over the years the Company has granted financial support to numerous research projects, the results of which have been published in the scientific literature. Three international symposia were held on the initiative of the Board with the aim of improving international exchange in this field of research.

During the last few years there has been a noticeable increase in research activity aimed at elucidating how nicotine or smoking interacts with nervous mechanisms. The Board therefore took the initiative of arranging a mini-symposium on the effects of nicotine on nervous functions held in Stockholm on 29 November 1978. The participants were recipients of grants from the Swedish Tobacco Company engaged in this type of research at first

hand, but in order to cover the subject more completely a few guest lecturers were invited. Thus Dr Boleslaw Srebro of the University of Bergen, Norway, made a survey of central cholinergic pathways and Dr Edith Heilbronn from the National Defence Research Laboratories, Stockholm, gave an outline of cholinergic receptors. The participants expressed the desire to make the symposium accessible to an international public by publishing the proceedings in English. The Swedish Tobacco Company acknowledges with gratitude the co-operation of its grantees and their co-workers towards the production of this publication.

L. Hjern

*Secretary of the Medical Advisory Board  
of the Swedish Tobacco Company*

Symposium on Chemical and Biological Problems Related to Smoking. *Acta Medica Scandinavica*, Supplement to Vol. 170 (1961).

Tobacco Alkaloids and Related Compounds, ed. U. S. von Euler. Pergamon Press, 1965.

Nicotine Workshop.—Abstracts in *Beiträge zur Tabakforschung* 8: 286-92 (1976).



## Assessment of cholinergic neuronal activity In the brain

AGNETA NORDBERG and ANDERS SUNDWALL

Department of Pharmacology University of Uppsala, and Department of Clinical Pharmacology  
Karolinska Institute Huddinge Hospital Sweden

**ABSTRACT:** Endogenous acetylcholine (ACh) ACh turnover and high affinity choline uptake (H.A. Ch uptake) have all been considered valuable markers for cholinergic neural activity. The present communication compares the markers regarding regional differences in the brain of normal mice as well as in mice treated with oxotremorine (OT) nicotine and sodium pentobarbital. A relationship is obtained between *in vivo* turnover values for 5 different brain regions and *in vitro* H.A. Ch uptake. Pretreatment with OT and sodium pentobarbital produces specific regional changes in both turnover and H.A. Ch uptake. However both drugs produce shifts of the correlation lines due to uptake being less affected than turnover. Atropine completely prevented the effects of OT regarding turnover and H.A. Ch uptake. A convulsant dose of nicotine did not produce significant changes in any of the brain regions studied neither on turnover nor on H.A. Ch uptake.

Measurements of the activity of acetylcholinesterase (AChE) choline acetyltransferase (CAT) and of the transmitter acetylcholine (ACh) have been extremely valuable for the identification and investigation of cholinergic synapses. Especially the sensitive histochemical and microchemical methods for AChE have made it possible to obtain a very detailed picture of the regional distribution of cholinergic and/or cholinceptive neurons in the brain.

Used together with cell fractionation technology the methods quoted have given a fairly good idea not only about the localization but also the organization of cholinergic synapses in brain. Less information has been obtained regarding the dynamic aspects of cholinergic neuronal activity.

Since the early discovery (Richter & Crossland 1949) that anesthesia and natural sleep produce inverse changes of the steady state level of acetylcholine (ACh) in the brain steady state levels have been the most commonly used indicator of cholinergic neuronal activity (Giarman & Pepeu 1962) (Table 1).

Due to the limitation of steady state levels other possibilities have been investigated e.g. release of ACh from the exposed cortex or from the inner surfaces of the brain (MacIntosh & Oborn 1943

Beanl et al 1968 Hemsworth & Mitchell 1969 Szerb et al 1970 Aquilonius et al 1977).

Steady state levels of the precursors Ch and acetylcoenzyme A (acetyl CoA) have been less frequently analysed and only oxotremorine (OT) has been found to produce significant changes decrease in acetyl CoA and increase in Ch (Schubert et al 1966 Schubert et al 1969).

The pioneer work by Quastel and co-workers demonstrated the biosynthesis of ACh in brain minces and slices and that the glucose and the  $N^+$  concentrations in the incubation medium are influential on synthesis, storage and release. Supply of Ch did not seem to be critical in these systems (Mann et al 1939). Later MacIntosh and co-workers in the perfused superior cervical ganglion of the cat demonstrated that the supply of Ch in the perfusion medium is necessary for optimal biosynthesis of ACh during prolonged electrical stimulation of the preganglionic nerve (Birks & MacIntosh 1961).

Introduction of readily available radioactive Ch and glucose has given more detailed knowledge about biosynthesis, storage and release of ACh in nervous tissue in both *in vivo* and *in vitro* preparations.

Slices from brain cortex accumulate the Ch with a carrier mediated transport mechanism, and the Ch



Table 3 Endogenous ACh and Ch (nmol/g) in different mouse brain regions following 3 methods of sacrifice (Nordberg and Sundvall 1976)

	Acetylcholine			Choline	
	Dislocation of the spine	Microwave irradiation		Microwave irradiation	
		Whole body 7 s	Head 0.25	Whole body 7	Head 0.25
Cerebellum	3.7±0.38 (11)	4.6±0.97 (3)	5.2±1.05 (4)	39.0±4.49 (6)	18.6±3.37 (3)*
Medulla oblongata	19.8±1.07 (12)	20.8±3.01 (3)	29.1±1.10 (4)***	44.0±7.24 (5)	46.7±11.30 (3)
Midbrain	1.1±1.24 (14)	23.1±1.47 (5)	29.4±1.65 (4)*	29.3±4.76 (7)	41.3±7.20 (3)
Striatum	37.1±2.06 (14)	40.4±3.73 (5)*	75.3±1.84 (3)***	48.8±6.50 (6)	41.4±5.65 (4)
Hippocampus	15.8±0.82 (14)	17.1±1.77 (5)	20.8±2.25 (4)	43.8±5.37 (6)	27.4±7.63 (3)
Cortex	13.2±0.74 (12)	18.4±1.55 (5)*	4.2±1.52 (4)***	30.4±2.80 (6)	30.5±3.50 (4)

In an additional sample 70.3 nmol/g

In an additional sample 48.7 nmol/g.

M±S.E. (n) = number of experiments : P<0.05 \*\*P<0.01 \*\*\*P<0.001

brain one extremely labile which can only be visualised with very short enzyme inactivation time and one rather stable about 50% of the total pool which is not changed until after several minutes. Measurements of the radioactive ACh formed following intravenous injection of a tracer dose indi-

cate a third pool of newly synthesized ACh with an intermediate stability (Fig. 1). It is not known how these pools relate to the free, labile bound and stable bound pools demonstrated by subcellular fractionation.

Although these experiments indicate that the

#### BIOSYNTHESIS OF $^3\text{H}$ -ACh IN DIFFERENT BRAIN REGIONS AFTER INTRAVENOUS INJECTION OF $^3\text{H}$ -Me-Ch

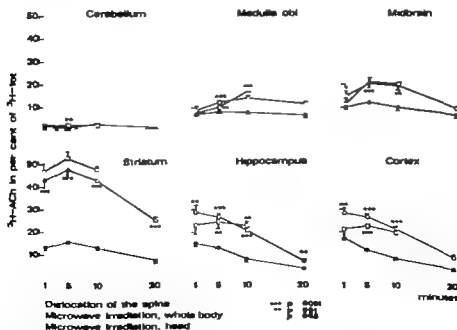


Fig. 1 10 nmol of  $^3\text{H}$ -Ch were injected i.v. and the animals were sacrificed by whole body microwave irradiation, by microwave irradiation of the head or by dislocation of the spine 1, 5, 10 or 20 min after injection. Each point represents the mean value of 3-5 experiments. Vertical bars indicate S.E. H-tot represent the total TCA extractable radioactivity (Nordberg & Sundvall 1976).

## TIME COURSE OF Ch AND ACh SPECIFIC RADIOACTIVITIES IN BRAIN REGIONS FOLLOWING TWO METHODS OF SACRIFICE

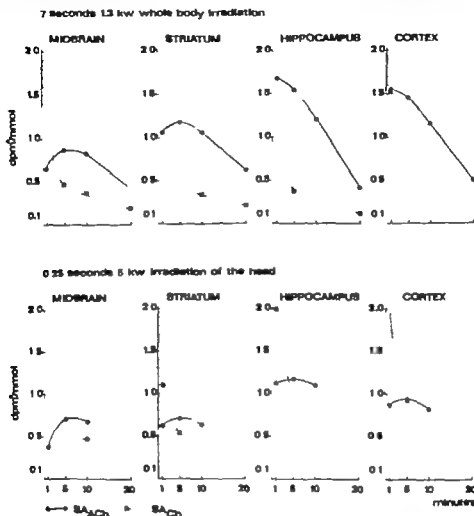


Fig. 2 Time course of the specific radioactivities of Ch and ACh following an intravenous pulse injection of  $^3\text{H}$ -Ch (15 nmol/mouse) (Nordberg and Sundwall 1976)

ACh in a particular brain region is not homogenous it was found when the specific radioactivity of ACh and Ch was plotted against time a relationship which was in agreement with the direct precursor product relationship between Ch and the formed ACh (Fig. 1)

Calculations of turnover = half life indicated rather great regional differences in the apparent turnover rate of ACh (Table 4). The highest turnover is in the striatum twice that in hippocampus and cerebral cortex which is about twice as high as in midbrain and medulla oblongata. In the cerebellum turnover is negligible.

A drug like sodium pentobarbital which earlier

had been found to increase the steady state concentration of ACh in whole brain and to decrease release from the exposed cortex was found to produce a rather specific effect on the turnover of ACh in the hippocampus and cortex leaving the other brain regions unaffected (Nordberg & Sundwall 1977). OT a muscarinic agonist which also produces increased steady state levels of ACh was found to produce a marked decrease in turnover in midbrain hippocampus and cortex (Nordberg 1978).

A convulsant dose of nicotine did not change the turnover in any of the regions (Nordberg & Sundwall 1979).

Table 4 Effect of sodium pentobarbital (60 mg  $\times$  kg  $^{-1}$  p) and oxotremorine (1 mg  $\times$  kg  $^{-1}$  p) on the apparent turnover rate (nmol  $\times$  g  $^{-1}$   $\times$  min  $^{-1}$ ) of ACh in different mouse brain regions

Brain region	Control	Sodium pentobarbital	Oxotremorine
Cerebellum	(7)	(2)	
Medulla oblongata	10	7	
Midbrain	11	9	5
Striatum	55	59	44
Hippocampus	21	6	7
Cort x	27	10	8

With the availability of radioactive Ch with extremely high specific radioactivity (60 Ci/nmol) it is possible to measure and compare turnover in extremely small regions (2 mg)

As mentioned above the sodium dependent high affinity Ch transport system appears to be located in cholinergic nerve terminals. By screening drugs known to affect turnover Kubar and co-workers have indicated that alterations of activity in cholinergic neurons *in vivo* are followed by parallel changes in sodium dependent high affinity Ch uptake *in vitro* (Atweh et al 1975). These findings have been confirmed and extended (Nordberg 1978; Nordberg & Sundwall 1979). In fact there appears to be a linear relationship between the turnover in different brain regions and sodium dependent high affinity Ch transport measured *in vitro* (Fig. 3). In mice pretreated with pentobarbital and OT the change in the rate of high affinity Ch uptake is related to the change in turnover. However turnover appears to be a more sensitive parameter since turnover was always affected to a greater degree than high affinity uptake. When the symptoms produced by OT were reversed by atropine the effect produced by OT on turnover and high affinity Ch uptake was abolished.

A convulsant dose of nicotine did not produce any effect on the high affinity Ch uptake (Nordberg & Sundwall 1979).

Even if there is yet much to be established regarding the kinetics of this high affinity uptake system for Ch the results which demonstrate a relationship between turnover and transport of Ch seem to support the view that this transport system is an important component in the regulation of the biosynthesis of ACh in the brain.

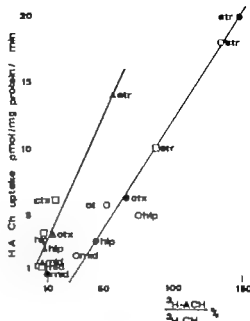


Fig. 3 Relationship between ACh turnover and H.A. Ch uptake in different brain regions.

○ control ▲ OT ● OT and atropine □ OT and methylatropine

The studies were financially supported by the Swedish Medical Research Council and the Swedish Tobacco Company.

## REFERENCES

- ATWEH S., SIMON J. R. & KUHAU, M. J. 1975. Utilization of sodium dependent high affinity choline uptake *in vitro* as a measure of the activity of cholinergic neurones *in vivo*. *Life Sci* 17: 1535-1544.
- AQUILONIS, S.-M., LUNDHOLM, B. & WINBLADH, B. 1972. Effects of some anticholinergic drugs on cortical acetylcholine release and motor activity in rats. *Eur J Pharmacol* 20: 224-230.
- BEANI, L., BIANCHI, C., SANTINOCETO, L. & MARCHETTI, P. 1968. The cerebral acetylcholine release in conscious rabbits with semi-permanently implanted epidural cups. *J. Neuropharmacol* 7: 469-481.
- BIRKS, R. I. & MACINTOSH, F. C. 1961. Acetylcholine metabolism of sympathetic ganglion. *Can J Biochem Physiol* 39: 787.
- DOMINO, E. F., VASKO, M. R. & WILSON, A. E. 1976. Mixed depressant and stimulant actions of acephane and their relationship to brain acetylcholine. *Lif Sci* 18: 361-376.
- GUYENET, P., LEFRESNE, P., ROSSIER, J., BEAUJOUIN, J. C. & GLOWINSKI, J. 1973. Inhibition by hemicholinium-3 of ( $^3$ H) acetylcholine synthesis and ( $^3$ H) choline high affinity uptake in rat striatal synaptosomes. *Molec Pharmacol* 9: 630-639.



- HANIN L, MASSARELLI R & COSTA E. 1970 Environmental and technical preconditions influencing choline and acetylcholine concentrations in rat brain. In: *Drugs and cholinergic mechanisms in the CNS*. Eds Heilbrunn & Winter. Almqvist & Wiksell, Stockholm, 33-54.
- HAUBRICH D R, WANG P R L, HERMAN R L & CLODY D E. 1975 Acetylcholine synthesis in rat brain: dissimilar effects of clozapine and chlorpromazine. *Life Sci* 17: 739-748.
- HEMSWORTH B A & MITCHELL J F. 1969 The characteristics of acetylcholine release mechanisms in the auditory cortex. *Br J Pharmacol* 36: 161-70.
- JENDEN D J. 1973 The use of gas chromatography-mass spectrometry to measure tissue levels and turnover of acetylcholine. In: *Advances in Biochemical Psychopharmacology* vol 7. Eds Costa & Holmstedt. Raven Press, New York, 69-81.
- KUHR, M J, SETHY V H, ROTH R H & AGHAJANIAN G K. 1973 Choline: selective accumulation by central cholinergic neurons. *J Neurochem* 20: 581-593.
- MACINTOSH F C & OBORIN P E. 1953 Release of acetylcholine from intact cerebral cortex. *Abstr Internat Physiol Congr* 19th 580.
- MANN P J G, TENNENBAUM M & QUASTEL J H. 1959 Acetylcholine metabolism in the central nervous system. *Biochem J* 33: 822-835.
- NORDBERG A & SUNDWALL, A. 1976 Biosynthesis of acetylcholine in different brain regions *in vivo* following alternative methods of sacrifice by microwave irradiation. *Acta Physiol Scand* 98: 307-317.
- NORDBERG A & SUNDWALL, A. 1977 Effect of sodium pentaobarbital on the apparent turnover of acetylcholine in different brain regions. *Acta Physiol Scand* 99: 336-344.
- NORDBERG A. 1978 Effect of oxotremorine on the apparent regional turnover of acetylcholine in mouse brain. *J Neurochem* 30: 383-389.
- NORDBERG A. 1978. Regional high affinity synaptosomal transport of choline in mouse brain—influence of oxotremorine treatment. *Life Sci* 23: 937-944.
- NORDBERG A & SUNDWALL, A. 1979 Unpublished observations.
- RACAGNI G, CHENEY D L, TRABUCCII M, WANG C & COSTA E. 1974 Measurement of acetylcholine turnover rate in discrete areas of rat brain. *Life Sci* 15: 1961-1975.
- RICHTER, D & CROSSLAND J. 1949 Variation in acetylcholine content of the brain with physiological state. *Am J Physiol* 159: 47-55.
- SAELENIS J K, SIMKE J P, ALLEN M P & CONROY C A. 1973 Some of the dynamics of choline and acetylcholine metabolism in rat brain. *Arch Int Pharmacodyn* 203: 305-312.
- SCHUBERTH J, SOLLENBERG J, SUNDWALL, A. & SÖRBO B. 1966 Acetylcholinesterase A in brain. The effect of centrally active drugs, insulin coma and hypoxia. *J Neurochem* 13: 819-822.
- SCHUBERTH J, SUNDWALL, A. & SÖRBO B. 1967 Relation between Na<sup>+</sup> K<sup>+</sup> transport and the uptake of choline by brain slices. *Life Sci* 6: 293-295.
- SCHUBERTH J & SUNDWALL, A. 1968 Differences in the subcellular localization of choline, acetylcholine and atropine taken up by mouse brain slices *in vitro*. *Acta Physiol Scand* 72: 65-71.
- SPARF B. 1973 On the turnover of acetylcholine in the brain—an experimental study using intravenously injected radioactive choline. *Acta Physiol Scand Suppl* 397: 7-47.
- STAVINOKA W B & RYAN L C. 1965 Estimation of the acetylcholine content of rat brain by gas chromatography. *J Pharmacol Exp Ther* 150: 231-235.
- STAVINOKA W B, WEINTRAUB S T & MODAK, A. T. 1973 The use of microwave heating to inactivate cholinesterase in the rat brain prior to analysis for acetylcholine. *J Neurochem* 20: 361-371.
- SZERB J C, MALIK H & HUNTER E G. 1970. Relationship between acetylcholine content and release in the cat's cerebral cortex. *Can J Physiol Pharmacol* 48: 780-790.
- TORU M & APRISON M H. 1966. Brain acetylcholine studies: a new extraction procedure. *J Neurochem* 13: 1333-1344.

# Biosynthesis and mammalian metabolism of nicotine

ÅKE PILOTTI

Department of Organic Chemistry Arrhenius Laboratory  
University of Stockholm S-10691 Stockholm Sweden

**ABSTRACT** The biosynthesis and mammalian metabolism of nicotine is summarized

Much effort has been devoted to the elucidation of the biosynthesis of tobacco alkaloids (1) and the gathered information regarding the biochemical precursors to nicotine [1] is presented in Fig. 1

In 1960 Dawson and coworkers showed that nicotinic acid (6) is the precursor to the pyridine ring (3, 4) and it was later established that the pyrrolidine ring is attached to C 3 of the nicotinic acid with loss of the carboxyl group from this position (5, 6)

The precursors to the pyrrolidine ring are derived from ornithine [1] and methionine as demonstrated by the incorporation of radioactivity into nicotine when labelled ornithine was fed to excised roots or intact tobacco plants (7, 8) When [ $^{14}\text{C}$ ] ornithine was fed to *N. glauca* or when *N. tabacum* was exposed to [ $^{14}\text{C}$ ,  $^{13}\text{C}$ ] carbon dioxide the isolated nicotine was found to have equal labelling at C 2 and C 5 (9) indicating the formation of a symmetric intermediate [2] On feeding the [ $^{14}\text{C}$ ]-*N*-methyl- $\Delta$ -pyrrolinium salt [5] all radioactivity of the isolated nicotine was found in the 2-position indicating that the randomisation of radioactivity between C 2 and C 5 in the first experiment must occur before the formation of [5]

As putrescine [2] was found to be incorporated into nicotine (10) the sequence ornithine [1] putrescine [2] *N*-methylputrescine [3] *N*-methyl- $\Delta$ -pyrrolinium salt [5] was proposed for the biosynthesis of the pyrrolidine ring of nicotine (8). The enzymes responsible for the conversions [1-4] have later been isolated from *N. tabacum* thus supporting the suggested sequence (11-13)

There has been some controversy whether this sequence via a symmetrical intermediate is major or minor route (14-16) Later results (9, 17-19)

support the pathway given in Fig. 1 which seems to be well established

Dawson found a much lower incorporation of radioactivity into nicotine after administration of [ $6\text{-}^3\text{H}$ ] nicotinic acid to excised roots of *N. tabacum* than after administration of the [ $2\text{-}^3\text{H}$ ], [ $4\text{-}^3\text{H}$ ] or [ $5\text{-}^3\text{H}$ ] labelled analogues (3). On feeding [ $6\text{-}^3\text{H}$ ] nicotinic acid ( $^{10}$ ) to intact tobacco plants Leete found an almost quantitative loss of tritium relative to incorporated  $^3\text{H}$ . Unreacted nicotinic acid retained most of the radioactivity indicating that the loss of tritium occurred in the biological activation of nicotinic acid which is a prerequisite for its condensation with the *N*-methyl- $\Delta$ -pyrrolinium salt. The mechanism in Fig. 2 was therefore suggested for the activation of nicotinic acid and the condensation to form nicotine (21) The 3,6-dihydronicotinic acid [7] however has not been isolated. Further the absolute configurations of the intermediates have not been established for the biosynthesis of nicotine (see below)

This mechanism starts with a stereospecific introduction of a hydrogen at C-6 of nicotinic acid giving [7] which via the carbanion [8] condenses with the pyrrolinium salt [5]. The product [9] is finally decarboxylated and the hydrogen originally present at C-6 is lost in a stereospecific dehydrogenation involving a hydride acceptor (2, 2)

In an elegant study of the stereochemistry involved in the biosynthesis of anatabine [10] (22) (Fig. 3) both rings of which are derived from nicotinic acid (23-24), it was suggested that the activation of nicotinic acid starts with a stereospecific reduction introducing  $^1\text{H}$  hydrogen at the pro-R position and that the final reduction involves the stereo-

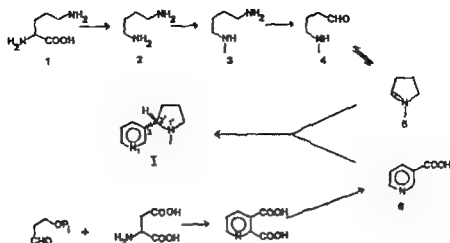


Fig 1 Biosynthesis of nicotine

specific dehydrogenation of the original pro-S-hydrogen

This should explain the complete loss of tritium from the pyridine ring and the corresponding retention of tritium in the piperidine ring.

These findings support the stereochemistry indicated in the intermediates involved in the nicotine biosynthesis

## METABOLISM OF NICOTINE

The present knowledge of the mammalian metabolism of nicotine has been reviewed in detail (24-27) and the combined results from the different research groups are summarised in Schemes 1a-1b (Fig 4) (28). The major metabolite of nicotine in most species and also the first mammalian metabolite of nicotine to be isolated (29) is cotinine.

The conversion of nicotine to cotinine is a two-step reaction involving two different enzyme sys-

tems and the structures IV, V and VI have been postulated as intermediates (30-32).

It has been suggested that 5-hydroxynicotine (IV) is first formed by C-oxidation involving cytochrome P-450 and that this metabolite is in equilibrium with the iminium ion V and the aminoaldehyde VI. The second step is mediated by the enzyme aldehyde oxidase (30).

The iminium ion (V) has been trapped in *in vitro* studies (32) and a study of the position of the equilibrium between IV, V and VI was recently performed (33) when the iminium ion became available through synthesis.

In a subsequent study (34) a purified aldehyde oxidase was used and it was demonstrated that the iminium ion was the most probable substrate for the enzyme. The alternative substrate VI was excluded as this form could not be detected in the equilibrium mixture (n.m.r.) (33). Furthermore, the expected product from an enzymatic oxidation of this amino-

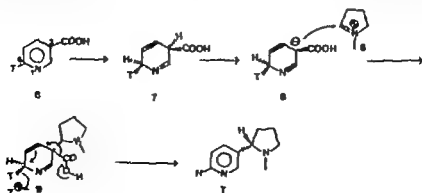


Fig 2 Activation of nicotinic acid and biosynthesis of nicotine

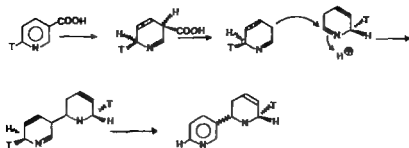


Fig 3 Biosynthesis of enatiabine

aldehyde would be the amino acid VII which does not cyclize to cotinine in an enzymic process (30).

It has been suggested that a metabolite of nicotine is responsible for the established protecting effect towards normally lethal doses of nicotine that can be attained through pretreatment with sublethal doses in various species (26, 35).

The iminium ion is thus of pharmacological interest as no other metabolite so far has been shown to be responsible for this effect (26, 27). As the muonium ion readily reacts with nucleophiles (31-36) it might also be responsible for the *in vitro* inhibition of cotinine production observed on addition of glutathione and cysteine (36). An adduct between the iminium ion and cysteine has indeed recently been observed (37).

*N*-Oxidation of natural *S*-nicotine is mediated by a microsomal flavoprotein and the two possible diastereoisomers of nicotine 1-oxide (*R*, *S*-*cis* and *S*, *S*-*trans*) are produced in varying proportions depending on the species being investigated (38-39). No further metabolism except reduction back to nicotine has been observed for these *N*-oxides, and the nicotine 1,1-dioxide has not been isolated as a metabolite.

Cotinine 1-oxide (XII) has been identified as a metabolite (40) but it has not been possible to establish whether its further metabolism occurs via X, XIII or via demethylcotinine 1-oxide-XIII.

Beside C- and N-oxidation, a third type of direct metabolism has been observed for nicotine, namely *N* methylation which gives nicotine isomethonium ion (II). No further metabolism of this ion into XI has been demonstrated although the latter has been isolated as a metabolite (41).

*N* Demethylation of nicotine and cotinine occurs in mammalian systems although conflicting results have been published (77). Demethylcotinine has been isolated from the urine of smokers (42) but the

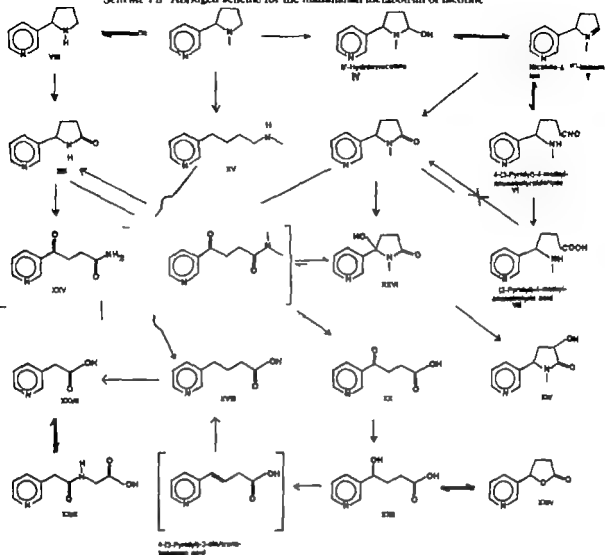
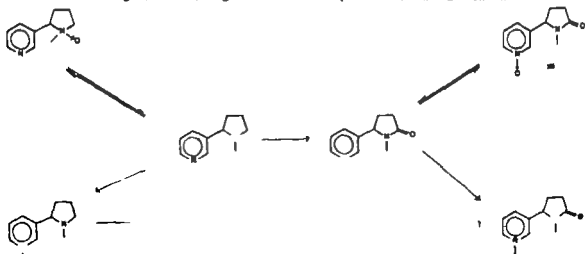
direct conversion X XIII has not been detected in humans given cotinine indicating that the alternative pathway I VIII XIII is prevailing (43).

In 1973 McKennis suggested that the *N*-de methylation of nicotine might proceed via a reactive methylene iminium ion ( $N=CH_2$ ) and such an intermediate was in fact later trapped in an *in vitro* experiment (44). All remaining metabolites of nicotine are believed to be formed from cotinine (X) or demethylcotinine (XIII) via the common metabolite XX, or direct from nicotine via metabolites XV and XVII. Hydrolysis of the lactam X (cotinine) gives the amino acid metabolite VII (45) but no further metabolism or enzymic ring closure back to nicotine has been established for this compound (see above).

Hydroxylation of X affords 3-hydroxycotinine (43) which is not known to give rise to any other metabolites or to 5-hydroxycotinine (XXVI, allo-hydroxycotinine). The two structures XXVI and IX are believed to be in equilibrium (76-78) and both have been isolated as metabolites of nicotine (76-78, 43-47, 48). However IR and  $^{14}C$  n.m.r. data have shown that only the cyclic isomer is present at physiological pH (26, 44, 49).

5-hydroxycotinine or its open form IX is metabolized further to the ketoacid XX (50) and the corresponding hydroxyacid (XXII) (51) via oxidative deamination and reduction of the ketone. In slightly acidic solutions the latter metabolite gives rise to the lactone XXIV spontaneously (26) and although this isomer has not been isolated as a nicotine metabolite it furnishes the remaining metabolites XVII and XXV II when fed to rats (26).

3-pyridylacetic acid (XXVII) is considered to be the ultimate metabolite of nicotine ( $5^3-^{13}C$ ), but its conjugate with glycine (XXIX) has been isolated (26). No evidence for the conversion of XXVII to nicotinic acid has been found.

*Scheme 1a* Abridged scheme for the mammalian metabolism of nicotine*Scheme 1b* Abridged scheme showing some and N-oxide intermediates in the mammalian metabolism of nicotine

Dihydrometanicotine (XV) presumably formed from nicotine by way of benzylic oxidation and ringopening to *N*-methyl-4-(3-pyridyl)-3-butenylamine (metanicotine) has been suggested as another source of the ultimate metabolite XXVII as both this acid and XVII could be isolated from urine of dogs to which XV had been administered (54)

## REFERENCES

- LEETE E 1973 *PhBip Morris Sci Symp Proc* 1st 91
- LEETE, E. 1976. 1st: Nicotine workshop 1974 Swed. Tobacco Company (ed. Å. Pilotti). (Abstract in *Beiträge zur Tabakforschung* 8: 290)
- DAWSON R. F. CHRISTMAN D. R. D'ADAMO A. SOLT M. L. & WOLF A. P. 1960. *J Amer Chem Soc* 82: 2628.
- SOLT M. L., DAWSON R. F. & CHRISTMAN D. R. 1960. *Plant Physiol* 35: 887
- YANG K. S. GHOSHON R. K. & WALLER G. R. 1963. *J Amer Chem Soc* 87: 4184
- SCOTT T. A. & GLYNN J. P. 1967. *Phytochemistry* 6: 503
- KISAKI, T. MIZUSAKI S. & TAMAKI E. 1966. *Arch Biochem Biophys* 117: 677
- LEETE, E. 1967. *J Amer Chem Soc* 89: 7081
- LEETE, E. 1976. *J Org Chem* 41: 3408
- LEETE, E. 1958. *J Amer Chem Soc* 80: 162.
- MIZUSAKI S. TANABE Y. NOUCHI M. & TAMAKI E. 1971. *Plant Cell Physiol* 12: 633
- MIZUSAKI S. TANABE Y. NOUCHI M. & TAMAKI E. 1972. *Phytochemistry* 11: 2757
- MIZUSAKI S. TANABE, Y. NOUCHI M. & TAMAKI E. 1973. *Plant Cell Physiol* 14: 103
- LEETE, E. 1976. *Biosynthesis* 4: 97
- LIEBMAN A. A. MORSENGH P. & RAPOPORT H. 1963. *J Amer Chem Soc* 87: 4399
- RUEPPEL, M. L., MUNDY B. P. & RAPOPORT H. 1974. *Phytochemistry* 13: 141
- ZIELKE, H. R. BYERRUM R. U. O'NEAL, R. M. BURNS, L. C. & KOEPE, E. 1967. *J Biol Chem* 242: 4737
- ZIELKE, H. R. REINKE, C. M. & BYERRUM R. U. 1968. *J Biol Chem* 244: 95
- LEETE E. 1971. *Chem Commun* 1524
- LEETE E. & LIU Y. Y. 1973. *Phytochemistry* 12: 593
- LEETE, E. 1977. *Biorganic Chem* 6: 273
- LEETE E. 1978. *J Chem Soc Chem Commun* 610
- LEETE, E. & SLATTERY S. A. 1976. *J Amer Chem Soc* 98: 6326.
- LARSON P. S. HAAG H. B. & SILVETTE, H. 1961. *Tobacco: Experimental and Chemical Studies*, Williams and Wilkins, Baltimore, Maryland
- McKENNIS H. In: *Tobacco Alkaloids and Related Compounds* (ed. U. S. von Esler) 1964 p. 53. Pergamon Press, Oxford
- McKENNIS H. I. Nicotine workshop 1974 Swed. Tobacco Company Stockholm (ed. Å. Pilotti) See reference 2.
- GORROD J. & JENNER, P. 1975. *Essays in Toxicology* 6: 35
- PILOTTI Å. ENZELL, C. R. McKENNIS H. J. & BOWMAN E. R. 1976. *Beiträge zur Tabakforschung* 8: 339
- McKENNIS H. TURNBULL, L. B. & BOWMAN E. R. 1957. *J Amer Chem Soc* 79: 6342.
- HUCKER, H. D. GILLETTE, J. R. & BRODIE, B. B. 1960. *J Pharmacol Exp Ther* 129: 94
- MURPHY P. J. 1973. *J Biol Chem* 248: 2796
- HILL, D. L. LASTER R., Jr & STRUCK, R. F. 1972. *Cancer Res* 32: 658.
- BRANDÄNGE, S. & LINDBLOM L. T. unpublished
- BRANDÄNGE S. & LINDBLOM L. 1979. *Acta Chem Scand B* 33: 187
- BARRASS B. C. BLACKBURN J. W. BRIMBLECOMBE R. W. & RICH P. 1969. *Biochem Pharmacol* 18: 2145
- GORROD J. W. JENNER, P. KEYSELL, G. R. & BECKETT A. H. 1971. *Chem-Biol Interact* 3: 269
- BRANDÄNGE S. & LINDBLOM, L. Unpublished results
- BOOTH, J. & BOYLAND E. 1970. *Biochem Pharmacol* 19: 733
- JENNER P. GORROD J. W. & BECKETT A. H. 1973. *Xenobiotica* 3: 573
- DAGNE, E. & CASTAGNOLI N. 1972. *J Med Chem* 15: 840
- McKENNIS H. TURNBULL, L. B. & BOWMAN E. R. 1963. *J Biol Chem* 238: 719
- BOWMAN E. R. TURNBULL, L. B. & McKENNIS H. 1959. *J Pharmacol Exp Ther* 127: 92.
- BOWMAN E. R. & McKENNIS H. 1962. *J Pharmacol Exp Ther* 135: 306
- NGUYEN T. L. ORUENKE, L. & CASTAGNOLI N. J. 1976. *J Med Chem* 19: 1168
- McKENNIS H. TURNBULL, L. B. & BOWMAN E. R. 1958. *J Amer Chem Soc* 80: 6397
- NGUYEN T. L. Ph.D. Thesis, University of California, San Francisco 1977
- McKENNIS H. J. BOWMAN E. R. QUIN L. D. & DENNEY R. C. Abstract of the American Chemical Society Meeting, New York, September 1966.
- DAGNE E. Ph.D. Thesis, University of California, San Francisco 1972.
- BRANDÄNGE, S. LINDBLOM, L. NISHIDA T. & PILOTTI Å. Unpublished results
- McKENNIS H. WADA E. BOWMAN E. R. & TURNBULL, L. B. 1961. *Nature* 190: 910.
- McKENNIS H. SCHWARTZ, S. L. TURNBULL, L. B. TAMAKI E. & BOWMAN E. R. 1964. *J Biol Chem* 239: 3981
- McKENNIS, H. BOWMAN E. R. & TURNBULL, L. B. 1961. *Proc Soc Exp Biol Med* 107: 143
- McKENNIS H. SCHWARTZ, S. L. & BOWMAN E. R. 1964. *J Biol Chem* 239: 3990.
- MEACHAM R. H. BOWMAN E. R. & McKENNIS H. 1977. *J Biol Chem* 247: 902.



## Studies of muscarinic and nicotinic binding sites in brain

AGNETA NORDBERG and CHRISTER LARSSON

Department of Pharmacology University of Uppsala, Sweden

**ABSTRACT** Binding properties for the muscarinic antagonist quinuclidinyl benzilate (QNB), the nicotinic antagonist tubocurarine and nicotine in crude synaptosomal fractions from mouse and rat brain have been studied. Marked regional differences in number of binding sites for labelled QNB ( $^3\text{H}$ -QNB) were found with high concentration in the stratum corneum and a low concentration in the cerebellum. The specific binding sites for labelled tubocurarine ( $^3\text{H}$ -tubocurarine) showed less regional variation. An increased number of muscarinic binding sites (with no change in receptor affinity) was found in rats following long-term treatment with barbital.

The introduction of high affinity ligands of high specific radioactivities has accelerated the receptor binding studies in brain during recent years. For studies of the muscarinic receptor several antagonists (atropine, propylbenzylcholine, methylscopolamine, quinuclidinyl benzilate) and agonists (oxotremorine-M, pilocarpine) have been used as ligands (Yamamura & Snyder 1974, Burgen & Huley 1975, Birdsall et al 1978, Hulme et al 1978 and Kloog & Sokolovsky 1978). Concerning the nicotinic receptor most studies have been performed in electric eel and peripheral nervous tissue and very little in brain.  $\alpha$ -Bungarotoxin, a neurotoxin which is known to act as an irreversible antagonist of nicotinic receptors in electric fish and skeletal muscle, has recently also been used for nicotinic receptor binding studies in brain. The evidence for and against  $\alpha$ -bungarotoxin as a nicotinic receptor ligand however has recently been discussed by Schmidt (1977) and Morley et al (1979).

In the present study we have measured both muscarinic and nicotinic binding sites in brain. For the studies of muscarinic binding sites the antagonist quinuclidinyl benzilate (QNB) was used as radiolabelled ligand and for the nicotinic receptor binding studies the antagonist tubocurarine and nicotine itself were used. The regional distribution of muscarinic and nicotinic binding sites in brain are compared. In addition some data obtained in collaboration with Dr G. Wahlström are presented

indicating an increased number of muscarinic binding sites in brain in the abstinence following long-term forced oral barbital administration.

### MATERIALS AND METHODS

**Binding assays.** A crude synaptosomal fraction (P fraction) (Gray & Whittaker 1962) was prepared from different parts of mouse and rat brain. The P fraction was diluted to protein concentration of 1-4 mg/ml. 100  $\mu\text{l}$  of the P fraction were added to 900  $\mu\text{l}$  N KPO buffer (pH 7.4) containing tritium labelled ligand and incubated for 2-60 min at 0-25°C. The incubation was terminated by cooling the samples to 0°C in ice followed by centrifugation in microfuge<sup>®</sup> (Beckman) for 4 min. The tip of the tubes were cut and the pellet dissolved in solvent/hexane (1:3) overnight (Terenius & Wahlström 1975). 14 ml scintillation mixture containing toluene, methanol and Permablend were added and the radioactivity was measured by liquid scintillation. Specific binding of  $^3\text{H}$ -QNB,  $^3\text{H}$ -tubocurarine and  $^3\text{H}$ -nicotine were calculated by subtracting the value for non-specific binding in presence of 100  $\mu\text{M}$  oxotremorine (+) tubocurarine or nicotine from the total binding in absence of unlabelled ligand. The protein content of the P fraction was measured (Lowry et al. 1951) and bovine albumin was used as standard.

**Radiolabelled ligand.** [ $^3\text{H}$ ]Tubocurarine chloride dextro (16.48 Ci/mmol) was purchased from New England Nuclear.

[ $^3\text{H}$ ]Nicotine *d*-betaric (3 Ci/mmol) was purchased from the Radiochemical Center, Amersham.

[ $^3\text{H}$ ]Quinuclidinyl benzilate (16 Ci/mmol) was purchased from the Radiochemical Center, Amersham.

**Chronic barbital treatment.** Male Sprague-Dawley rat were chronically receiving sodium barbital (0.31 mg/ml) in



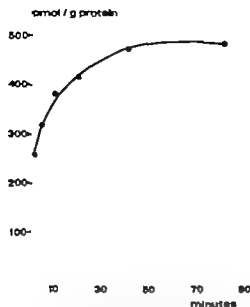


Fig. 1 Specific binding of H-QNB as function of time of incubation. 0.4 nM  $^3$ H-QNB (racemate) were incubated with crude synaptosomal (P) fractions from the mouse cortex for different times (2–80 min). Each point represents the mean value of 3 experiments. ● specific binding; ▲ unspecific binding.

their drinking fluid. The daily consumption of barbital was about 200 mg/kg/day. After about 40 weeks of treatment the barbital solution was replaced by water. One group of animal were killed at the time of withdrawal of the barbital solution and two other groups of animal were killed after 3 and 12 days of abstinence. Abstinence convulsions were recorded. For further detail regarding the chronic treatment see Wahlström (1974) and Nordberg et al. (1979).

## RESULTS AND DISCUSSION

Fig. 1 shows that the specific binding of H-QNB to a crude synaptosomal fraction (P fraction) is maximal after 40 min of incubation at  $25^{\circ}\text{C}$ . The specific binding is about 90% of the total binding. Incubation with different concentrations of H-QNB indicates a saturation of the binding at about 1 nM H-QNB (Fig. 2). Muscarinic agonists and antagonists can displace H-QNB from its binding sites. Inhibition curves for atropine and oxotremorine are shown in Fig. 3.  $\text{IC}_{50}$  values of  $5 \times 10^{-6}$  M for atropine and  $7 \times 10^{-5}$  M for oxotremorine are obtained (Fig. 3). Thus, hundred time difference in  $\text{IC}_{50}$  value is found between the agonist and an antagonist.

The distribution of H-QNB binding sites in brain is shown in Table 1. The highest content of binding

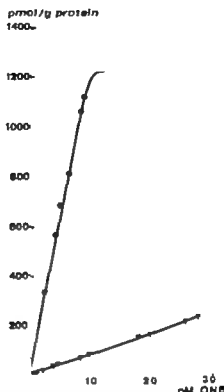


Fig. 2 Specific binding of H-QNB as function of H-QNB concentration. Different concentrations of H-QNB were incubated with P<sub>1</sub> fractions from the mouse cortex at  $25^{\circ}\text{C}$  for 60 min. Each point represents the mean value of 3 experiments. ● specific binding; ▲ unspecific binding.

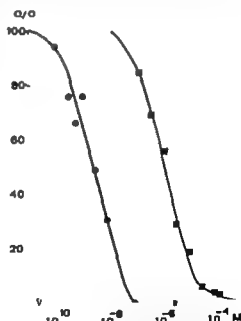


Fig. 3 Inhibition by atropine (●) and oxotremorine (■) of the specific binding of  $^3$ H-QNB (0.4 nM racemate) to P<sub>1</sub> fraction from the mouse cortex. Each point represents the mean value of 3 experiments.

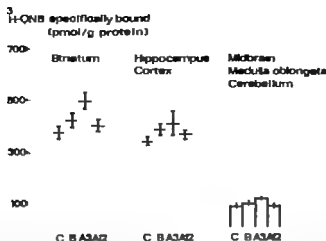


Fig. 4 Effect of long term barbiturate treatment on number of  $^3\text{H}$ -QNB binding sites in different regions of rat brain. C=control B=barbiturate until sacrifice A3=abstinence for 3 days, A12=abstinence for 12 days. Each column represents the mean value from 6-9 animals (Nordberg et al. (1979)).

sites is found in the cortex striatum intermediate in the hippocampus midbrain medulla oblongata and lowest in the cerebellum. About the same number of binding sites are found in the striatum and cortex although the content of endogenous acetylcholine (ACh) ACh turnover and high affinity synaptosomal choline (Ch) uptake are 2-3 times higher in the striatum in comparison with the cortex (Nordberg 1977 1978). Thus there seems to be no obvious relationship between ACh turnover and number of muscarinic binding sites.

Chronic treatment with barbiturate can increase the number of muscarinic binding sites in brain

Table 1 Regional distribution of  $^3\text{H}$ -QNB and  $^3\text{H}$ -tubocurarine binding sites in brain

Crude synaptosomes from mouse brain were incubated with  $^3\text{H}$ -QNB (4 nM racemate) for 60 min at 25°C. Crude synaptosomes from rat brain were incubated with  $^3\text{H}$ -tubocurarine (3 nM) for 15 min at 25°C. Mean values of 2-3 experiments.

Brain region	$^3\text{H}$ -QNB binding (pmol/g protein)	$^3\text{H}$ -tubocurarine binding (pmol/g protein)
Cortex	461	144
Striatum	56	104
Hippocampus	471	99
Midbrain	320	1
Hypothalamus		133
Medulla oblongata	222	140
Cerebellum	94	99

(Nordberg et al. 1979). Fig. 4 shows the results from an experiment where rats had been treated for 40 weeks. When the barbiturate solution was withdrawn (B) no significant effect on the number of  $^3\text{H}$ -QNB binding sites was found. 3 days later (A3) when abstinence convulsions were maximal a 30% higher number of  $^3\text{H}$ -QNB binding sites was found in the striatum and midbrain preparations. On the 12th day of abstinence the number of binding sites had returned to control level. A Scatchard plot of the binding data for the midbrain preparation showed an increase in  $B_{\text{max}}$  with 40% on the 3rd day of abstinence in comparison with control. No difference was found in the receptor affinity. Other studies indicate an increased turnover of ACh during the early part of the barbiturate abstinence (Nordberg & Wahlstrom 1977 1979). This is as far as we know the first study showing that chronic drug treatment can increase the number of muscarinic binding sites in brain.

Table 2.  $\text{IC}_{50}$  values for *t*-tubocurarine and nicotine competing with *H*-tubocurarine (3 nM) and *H*-nicotine (70 nM)

Labelled ligand	$\text{IC}_{50}$ $10^{-6}$ M	
	Tubocurarine	Nicotine
$^3\text{H}$ -tubocurarine	2	65
$^3\text{H}$ -nicotine	50	100

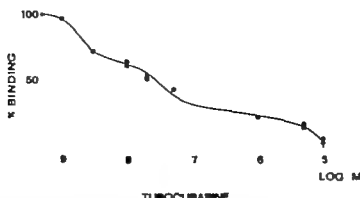


Fig. 5 Inhibition by tubocurarine of the specific binding of  $^3\text{H}$ -tubocurarine (3 nM) to P2 fractions from the mouse hippocampus. The incubation was performed at  $25^\circ\text{C}$  for 15 min. Each point represents the mean value of 3 experiments.

The binding of  $^3\text{H}$ -nicotine and  $^3\text{H}$ -tubocurarine to a P fraction from the hippocampus can be measured at  $0^\circ\text{C}$ . The  $^3\text{H}$ -nicotine binding is the same at  $0^\circ\text{C}$  and  $25^\circ\text{C}$  while the  $^3\text{H}$ -tubocurarine binding increases with 50% in the temperature range  $0^\circ\text{C}$  to  $25^\circ\text{C}$ . The specific binding for both ligands is about 30–40% of the total binding. A maximal specific binding is obtained after about 5 min of incubation.

The  $^3\text{H}$ -nicotine and  $^3\text{H}$ -tubocurarine binding can be blocked by increasing concentrations of unlabelled ligands. Table 2 shows  $\text{IC}_{50}$  values for nicotine and tubocurarine obtained after incubation with  $^3\text{H}$ -nicotine and  $^3\text{H}$ -tubocurarine. As can be seen in Table 2, tubocurarine is 25 times more potent in inhibiting  $^3\text{H}$ -tubocurarine binding in comparison with  $^3\text{H}$ -nicotine binding. For nicotine about the same  $\text{IC}_{50}$  value is obtained with both labelled ligands.

The specific binding of  $^3\text{H}$ -tubocurarine to crude synaptosomes increases linearly with increasing concentrations up to 10 nM (concentration range 1–10 nM). A Scatchard plot of the binding data indicates two different binding sites with a  $K_d$  of 1.5 and 14 nM respectively. Binding studies in brain using labelled  $\alpha$ -bungarotoxin or naja-naja sialenistoxin have shown the binding site. Eterovic & Bennett (1974) found a  $K_d$  of 3 nM using  $\alpha$ -bungarotoxin and Speth et al. (1971) a  $K_d$  of 0.7 nM with naja-naja toxin. The possibility of more than one bungarotoxin binding site has been discussed by Morley et al. (1979). Figure 5 shows the inhibition of  $^3\text{H}$ -tubocurarine binding by unlabeled tubocurarine at several different concentrations of unlabeled tubocurarine. As can be seen in Figure 5, the

inhibition curve is very flat and has an  $\text{S}$  shape which might indicate more than one binding site.

The distribution of  $^3\text{H}$ -tubocurarine binding sites in different parts of the brain is at present under investigation. Some recently obtained data are given in Table 1 and as can be concluded from the table the  $^3\text{H}$ -tubocurarine binding sites are more even distributed in brain in comparison with the  $^3\text{H}$ -QNB binding sites (Table 1).

The studies were financially supported by the Swedish Tobacco Company and the Swedish Medical Research Council.

## REFERENCES

- BIRDSALL, N. J. M., BURGEN A. S. V. & HULME E. C. 1978. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* 14, 723–736.
- BURGEN A. S. V. & HILEY C. R. 1974. The use of an alkylating antagonist in investigating the properties of muscarinic receptors. In: *Cholinergic mechanisms* (ed. P. G. Waser) pp. 381–385. Raven Press, New York.
- ETEROVIC V. A. & BENNETT E. L. 1974. Nicotinic cholinergic receptor in brain detected by binding of  $\alpha$ -[ $^3\text{H}$ ]bungarotoxin. *Biochem. Biophys.* 36, 346–353.
- GRAY E. O. & WHITTAKER V. P. 1966. The isolation of nerve endings from brain: an electron microscopic study of cell fragment derived by homogenization and centrifugation. *J. Anat. (Lond.)* 96, 79–88.
- HULME E. C., BIRDSALL, N. J. M., BURGEN A. S. V. & MEHTA J. 1978. The binding of antagonists to brain muscarinic receptors. *Mol. Pharmacol.* 14, 737–750.
- KLOOG Y. & SONOLOVSKY M. 1978. Muscarinic binding in mouse brain receptor sites. *Biochem. Biophys. Res. Comm.* 81, 710–717.
- LOWRY O. H., ROSENBRUGH N. J., FARR A. L. & RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. *Biol. Chem.* 193, 265–275.

- MORLEY B J, KEMP G E. & SALVATERRA, F. 1979. Bungarotoxin binding sites in the CNS. *Life Sci* 4 859-871.
- NORDBERG A. 1977. Apparent regional turnover of acetylcholine in mouse brain—methodological and functional aspects. *Acta Physiol Scand Suppl.* 445 1-51.
- NORDBERG A. 1978. Regional high affinity synaptic transport of choline in mouse brain—Influence of oxotremorine treatment. *Life Sci* 3 937-944.
- NORDBERG A. & WAHLSTRÖM G. 1977. Effect of long-term forced oral barbitol administration on endogenous acetylcholine in different regions of rat brain. *Eur J Pharmacol* 43 237-242.
- NORDBERG A. & WAHLSTRÖM G. 1979. Regional biosynthesis of acetylcholine in brains following forced oral chronic barbitone treatment to rat. *J Neurochem* 32 371-378.
- NORDBERG A, WAHLSTRÖM G. & LARSSON C. 1979. Increased number of muscarinic binding sites in brain following chronic barbiturate treatment to rat. *Life Sci*. Submitted for publication.
- SCHMIDT J. 1977. Drug binding properties of an  $\alpha$ -Bungarotoxin-binding component from rat brain. *Mol Pharmacol* 13 283-290.
- SPETH R. C., CHEN F. M., LINDSTRÖM J. M., KOBAYASHI R. M. & YAMAMURA H. L. 1977. Nicotinic cholinergic receptors in rat brain identified by [ $^{125}$ I] Naja naja splanchnic  $\alpha$ -toxin binding. *Brain Research*, 150-155.
- TERENIUS, L. & WAHLSTRÖM A. 1975. Search for an endogenous ligand for the opiate receptor. *Acta Physiol Scand* 94 74-81.
- WAHLSTRÖM G. 1974. Withdrawal in the rat after long-term forced oral barbitol administration. *Acta Pharmacol Toxicol* 35 131-144.
- YAMAMURA H. T. & SNYDER, S. H. 1974. Muscarinic cholinergic binding in rat brain. *Proc Nat Acad Sci* 71 1725-1729.



## Effects of neonatal nicotine administration on the postnatal development of central noradrenaline neurons

■ JONSSON and H. HALLMAN

Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden

**ABSTRACT** The effects of neonatal nicotine and/or 6-hydroxydopamine (6-OH-DA) treatment on the postnatal development of central noradrenaline (NA) neurons have been investigated using neurochemical and histochemical techniques. Nicotine was found to produce an increase of  $^3\text{H}$ -NA uptake and endogenous NA in the cerebral cortex and pons-medulla, which was most pronounced at the age of one week. These parameters were normalized in the adult stage. Neonatal nicotine treatment was also found to partially counteract the 6-OH-DA induced alteration of the development of the *locus coeruleus* NA system. The NA deafferentation produced by 6-OH-DA in the cerebral cortex and the spinal cord was thus counteracted by nicotine treatment. The results suggest that neonatal nicotine administration has a growth stimulatory effect on the early postnatal development of central NA neurons.

The central catecholamine (CA) neurons are known to appear early in gestation in many species including man. The CA perikarya are developed at birth while their nerve terminal projections continue to develop postnatally and reach adult nerve density about one month after birth (see Jonsson 1976). Previous studies have shown that the postnatal development of the central noradrenaline (NA) neurons is normally highly ordered and strictly programmed from a structural standpoint indicating a high degree of intrinsic growth regulation. However, very little is known as to mechanisms involved in governing growth processes and the development of the regional innervation pattern of central NA neurons. In view of the findings that the preganglionic cholinergic input is of great importance for the development of the end organ innervation by the postganglionic sympathetic neurons in the peripheral nervous system (Black & Mytilineou 1976) it was considered of interest to investigate whether or not nicotine can affect the postnatal development of central NA neurons.

### MATERIALS AND METHODS

Albino rats (Sprague-Dawley) were used. The drug treatments were started on the day of birth and four groups of animals were studied: A Controls; B Nicotine (3-4x2

mg/kg s.c. 24 h intervals; C 6-OH-DA (100 mg/kg s.c., within 4 h after birth); D 6-OH-DA (100 mg/kg s.c.) and nicotine (3-4 mg/kg s.c., 24 h intervals). The first acetone injection was given 3-4 h after the 6-OH-DA administration. The animals were killed by decapitation.  $^3\text{H}$ -NA uptake (0.05  $\mu\text{M}$ , 5 min) was measured *in vitro* in homogenates according to Jonsson et al. (1974). Endogenous CA concentrations were determined by using high pressure liquid chromatography with electrochemical detection (Keller et al. 1976). The fluorescence histochemical method of Falck-Hillarp for the demonstration of biogenic monoamines was utilized to study the fluorescence morphology of the CA neurons (Falck et al. 1962; Corrodi & Jonsson 1967).

### RESULTS AND DISCUSSION

Neonatal nicotine treatment was found to produce a 25-30% increase in  $^3\text{H}$ -NA uptake *in vitro* and endogenous NA levels in the neocortex one week after the treatment while there was a normalization of these parameters in the adult stage. Similar effects were observed in the pons-medulla although less pronounced. These data indicate that nicotine has a growth stimulatory effect on the NA nerve terminals although not persistent which may be due to the short period of nicotine administration. Analysis of the effects of neonatal nicotine and/or 6-OH-DA treatment on the regional CA concentrations in the adult stage showed that nicotine treat-



# Mecamylamine Induced blockade of nicotine Induced inhibition of gonadotrophin and TSH secretion and of nicotine induced increases of catecholamine turnover in the rat hypothalamus

KURT ANDERSSON KJELL FUXE PETER ENEROTH  
JAN-ÅKE GUSTAFSSON<sup>2</sup> and LUIGI F. AGNATI

Departments of <sup>1</sup>Histology and <sup>2</sup>Medical Chemistry Karolinska Institutet, Stockholm, and <sup>3</sup>the Hormone Laboratory Department of Obstetrics and Gynecology Karolinska Hospital Stockholm, Sweden

**ABSTRACT** Nicotine treatment of normal male rats and of ovariectomized female rats results in increases of dopamine (DA), noradrenaline (NA) and adrenaline (A) turnover in the hypothalamus especially within the median eminence. These effects may in part mediate the inhibition of gonadotrophin and TSH secretion produced by nicotine.

Previous work has indicated that in the normal male rat and in the ovariectomized female rat, nicotine may reduce LH and prolactin secretion via an activation of the tubero-infundibular dopamine (DA) neurons to the lateral and medial palisade zone of the median eminence respectively (Fuxe et al 1977, Eneroth et al 1977). In order to evaluate if these changes are related to the activation by nicotine of central nicotine-like cholinergic receptors it has in the present study been analysed if the ganglion blocking agent mecamylamine can block the hormonal effects and catecholamine (CA) turnover effects of nicotine within the hypothalamus.

## MATERIALS AND METHODS

Normal male specific pathogen free Sprague-Dawley rats and 1 month and 3 month ovariectomized female specific pathogen free Sprague-Dawley rats have been used. Nicotine as injected alone or together with mecamylamine, the dose and time schedule shown in legend to Table 1. A possible change in CA stores following treatment with the tyrosine hydroxylase inhibitor  $\alpha$ -methyl-tyrosine methyl ester (H 44/68 250 mg/kg p.o. 1 or 2 h before killing) (Anders et al 1969). In the regional analysis of CA stores in the various parts of the hypothalamus, Palck-Hillarp technique for the demonstration of the cellular localization of CA was used in combination with quantitative microfluorimetry (see Fuxe et al 1977). In the biochemical analysis of the CA stores

in the whole rat hypothalamus, high pressure liquid chromatography was used in combination with electrochemical detection (Keller et al 1976). Determinations of serum levels of LH, FSH, prolactin and TSH were obtained by means of radioimmunoassay using kits for the assay of LH, FSH, prolactin and TSH which were kindly supplied by The National Institute for Arthritis, Metabolism and Digestive Diseases (NIAMDD) rat pituitary hormone distribution program (see Fuxe et al 1977, Eneroth et al 1977).

## RESULTS

As seen in Table 1, nicotine treatment in repeated doses of 1 mg/kg produces a significant depletion of the CA stores in the various parts of the median eminence of the ovariectomized female rat. The experiment with H 44/68 revealed an enhancement by nicotine of the H 44/68 induced depletion of CA stores in the various parts of the median eminence. In the forebrain, on the other hand, nicotine could not deplete the DA stores in tuberculum olfactorium, nuc. accumbens and nuc. caudatus. In the nuc. accumbens nicotine reduced the H 44/68 induced depletion of the DA stores, while nicotine did not influence the other two regions. The results obtained on hormone levels in ovariectomized female rat are summarized in Table 2. Nicotine in the same dose range as above produced marked inhibition of prolactin, LH, FSH and TSH serum



Table 1 Effect of nicotine on CA fluorescence and H 44/68 induced CA fluorescence disappearance in various regions of the 3 month ovariectomized female rat

Nicotine (2 mg/kg, i.p.) was given 4 times with 30 min intervals, the animals being killed 30 min after the last injection. H 44/68 (250 mg/kg, i.p.) was given 2 h before decapitation in the saline treated group and 5 min after the first injection of nicotine in the nicotine treated group. Means  $\pm$  S.E. in per cent of saline treated group means. Number of animals in parenthesis. In the statistical analysis Mann-Whitney U-test was used.  $P < 0.05$   $P < 0.01$   $P < 0.001$  SEL = subependymal layer of the median eminence; MPZ = medial palisade zone of the median eminence; LPZ = lateral palisade zone of the median eminence; TO = tuberculum olfactorium; ACC = nucleus accumbens; CAUD = nucleus caudatus.

Treatment	Dose (mg/kg)	SEL	MPZ	LPZ	TO	ACC	CAUD
Saline		100 $\pm$ 9 (7)	100 $\pm$ 3 (7)	100 $\pm$ 6 (7)	100 $\pm$ 5 (7)	100 $\pm$ 7 (7)	100 $\pm$ 8 (7)
Nicotine	4 $\times$ 2	64 $\pm$ 4 (7)	80 $\pm$ 4 (7)	84 $\pm$ 4 (7)	92 $\pm$ 7 (7)	95 $\pm$ 6 (7)	104 $\pm$ 9 (7)
Saline + H 44/68		69 $\pm$ 6 (7)	43 $\pm$ 4 (7)	43 $\pm$ 2 (7)	63 $\pm$ 6 (5)	57 $\pm$ 3 (7)	72 $\pm$ 3 (7)
Nicotine + H 44/68	4 $\times$ 2	43 $\pm$ 3 (7)	33 $\pm$ 3 (7)	32 $\pm$ 2 (7)	60 $\pm$ 6 (7)	68 $\pm$ 4 (7)	77 $\pm$ 4 (7)

Table 2 Effect of mecamlamine on the nicotine + H 44/68 induced changes in PRL, LH, FSH and TSH levels of the 1 month ovariectomized female rat

Ovariectomy was performed 1 month before killing. Nicotine (2 mg/kg, i.p.) was given immediately before H 44/68 (250 mg/kg, i.p.) 1 h before killing and 30, 60 and 90 min after H 44/68. Mecamlamine (1 mg/kg, i.p.) was given 1 h before and 1 h after H 44/68. Means  $\pm$  S.E. of hormone levels are given in per cent of saline + H 44/68 treated group means. Number of animals in parenthesis. Statistical analysis according to Wilcoxon, one-way classification, comparing all possible pairs of treatments.  $P < 0.05$   $P < 0.01$ . In the saline + H 44/68 treated group the serum levels of TSH are 1223  $\pm$  138 ng/ml, the serum prolactin levels were 265  $\pm$  37 ng/ml, the serum LH levels were 469  $\pm$  3 ng/ml and the serum FSH levels were 2197  $\pm$  64 ng/ml.

Treatment	Dose (mg/kg)	PRL (%)	LH (%)	FSH (%)	TSH (%)
Saline + H 44/68		100 $\pm$ 14 (7)	100 $\pm$ 7 (7)	100 $\pm$ 3 (7)	100 $\pm$ 11 (7)
Nicotine + H 44/68	4 $\times$ 2	14 $\pm$ 1 (5)	43 $\pm$ 7 (5)	79 $\pm$ 3 (5)	43 $\pm$ 6 (5)
Mecamlamine + nicotine + H 44/68	2 $\times$ 1 4 $\times$ 2	35 $\pm$ 7 (6)	65 $\pm$ 6 (6)	92 $\pm$ 6 (6)	77 $\pm$ 10 (6)
Mecamlamine + H 44/68	2 $\times$ 1	100 $\pm$ 5 (7)	91 $\pm$ 10 (7)	88 $\pm$ 6 (7)	157 $\pm$ 13 (7)

Table 3 Reduction by mecamlamine of nicotine induced increases in the H 44/68 induced depletion of NA, A and DA stores in the hypothalamus of the normal male rat

Nicotine (2 mg/kg s.c.) was given 2 times with 30 min intervals, the first dose given immediately before H 44/68 (250 mg/kg, i.p.) 1 h before decapitation. Mecamlamine (1 mg/kg s.c.) was given immediately before the first nicotine or saline injection. Means  $\pm$  S.E. in per cent of H 44/68 treated group means.  $n = 6$ . Statistical analysis according to Wilcoxon, one-way classification, comparing all possible pairs of treatments.  $P < 0.05$   $P < 0.01$ . NA 100% = 841  $\pm$  162 ng/g tissue; A 100% = 76  $\pm$  3 ng/g tissue; DA 100% = 263  $\pm$  23 ng/g tissue.

	NA (%)	A (%)	DA (%)
Saline + H 44/68	100 $\pm$ 6	100 $\pm$ 6	100 $\pm$ 9
Nicotine 2 $\times$ 2 mg/kg + H 44/68	38 $\pm$ 3	41 $\pm$ 1	39 $\pm$ 5
Mecamlamine 1 mg/kg + nicotine 2 mg/kg + H 44/68	87 $\pm$ 10	86 $\pm$ 5	103 $\pm$ 12
Mecamlamine 1 mg/kg + H 44/68	86 $\pm$ 10	103 $\pm$ 8	76 $\pm$ 10

levels in the presence of the tyrosine hydroxylase inhibitor Mecamylamine treatment with two doses of 1 mg/kg partly seemed to counteract the nicotine induced inhibition of gonadotrophin and TSH secretion. Mecamylamine alone did not by itself produce any significant changes in hormone secretion but there was a trend for an increase in TSH secretion following mecamylamine treatment in combination with tyrosine hydroxylase inhibition. The results obtained in the biochemical analysis of the CA stores in the whole rat hypothalamus following treatment with nicotine are shown in Table 3. It is seen that nicotine produces an enhancement of the H 44/68 induced depletion of the noradrenaline (NA), adrenaline (A) and DA stores in the normal male rat hypothalamus. The enhancement of the H 44/68 induced depletion of all the CA stores is blocked by pretreatment with mecamylamine in a dose of 1 mg/kg. Mecamylamine in doses of 3–10 mg/kg by itself induced an enhancement of the H 44/68 induced depletion of the DA and A stores in the hypothalamus. A similar but not significant effect was present within the NA systems.

## DISCUSSION

The present findings give further evidence that nicotine treatment can reduce the DA and NA stores within the median eminence and that it increases DA and NA turnover within this region. Mecamylamine pretreatment, as shown in the biochemical experiment resulted in a blockade of the nicotine induced increases of NA and DA turnover found within the hypothalamus. Thus evidence is given that the nicotine induced increases of DA and NA turnover are due to the activation of cholinergic nicotine-like receptor probably located within the hypothalamus. The discovery was also made that nicotine can increase A turnover within the hypothalamus, an effect which also is blocked by mecamylamine pretreatment.

Thus nicotine-like cholinergic receptors may also control the release of A from adrenergic terminals within the hypothalamus. Mecamylamine pretreatment could also in part counteract the nicotine induced inhibition of gonadotrophin and TSH secretion. It therefore seems possible that also the hormone changes induced by nicotine are related in part to stimulation of a cholinergic nicotine-like receptor. It is suggested that cholinergic nicotine-like receptors exist on the DA, NA and A terminals within the hypothalamus and that their activation by nicotine can result in inhibition of gonadotrophin and TSH secretion at least in the dose range used in the present analysis.

This work has been supported by grants from the Swedish Tobacco Co. and the Council for Tobacco Research, U.S.A. Inc. New York, U.S.A. Mecamylamine hydrochloride was generously supplied by Merck, Sharp & Dohme U.S.A.

## REFERENCES

- ANDÉN N.-E., CORRODI H. & FUXE, K. 1969 Turnover studies using metabolic inhibition. In: *Metabolism of amines in the brain* (ed. G. Hooper), pp. 38–47. Macmillan, London.
- ENEROTH P., FUXE, K., GUSTAFSSON J. Å., HÖKFELT T., LÖFSTRÖM A., SKETT P. & AGNATI L. 1977 The effect of nicotine on central catecholamine neurons and gonadotropin secretion. II. Inhibitory influence of nicotine on LH, FSH and prolactin secretion in the ovariectomized female rat and its relation to regional changes in dopamine and noradrenaline levels and turnover. *Med Biol* 55: 158–166.
- FUXE, K., AGNATI L., ENEROTH P., GUSTAFSSON J. Å., HÖKFELT T., LÖFSTRÖM A., SKETT P. & SKETT P. 1977 The effect of nicotine on central catecholamine neurons and gonadotropin secretion. I. Studies in the male rat. *Med Biol* 55: 148–157.
- KELLER, R. O.K.E., A. MEDFORD I. & ADAMS R. N. 1976. Liquid chromatographic analysis of catecholamines—routine assay for regional brain mapping. *Life Sci* 19: 995–1004.



## Effect of nicotine on single cell activity in the noradrenergic nucleus locus coeruleus

TORGY H. SVENSSON and GÖRAN ENGBERG

Department of Pharmacology, University of Göteborg, Göteborg, Sweden

**ABSTRACT** The effect of nicotine and various cholinergic and anticholinergic drugs on brain noradrenaline (NA) containing neurons in the locus coeruleus (LC) of the rat was studied utilizing single cell recording techniques and microiontophoretic drug application. Nicotine (10–40 µg/kg i.v.) caused a dose-dependent, short-lasting increase in the firing rate of NA neurons in the LC. When microiontophoretically applied, nicotine had no effect on LC neurons, which in contrast were activated by acetylcholine (ACh) and the muscarinic agonist, bethanechol. The stimulation of NA neurons in the LC by L-physostigmine was antagonized by scopolamine but not by methylscopolamine. Also microiontophoretically applied scopolamine specifically antagonized the NA-cell activation by ACh. Consequently nicotine probably causes an indirect activation of NA neurons in the LC. These cells seem to be equipped with excitatory cholinergic receptors of muscarinic but not nicotinic character. The LC activating effect of nicotine may be important for its arousal producing properties.

**Key words:** Nicotine, NA neurons, Locus coeruleus, muscarinic receptors, arousal

Previous experiments have shown that nicotine causes an increased release of <sup>3</sup>H noradrenaline (<sup>3</sup>H NA) into the effluent from the perfused cerebral ventricle of the cat (Hall and Turner 1972). The drug also increased the release of <sup>3</sup>H NA from slices of rat hypothalamus and cerebellum (Westfall 1974). Interestingly also physostigmine like oxotremorine seemed to activate brain NA mechanisms since it caused acceleration of the  $\alpha$ -methyl-p-tyrosine induced disappearance of brain catecholamines (CA) (Corrodi et al 1967) as well as increased the central CA synthesis rate (Andén & Wachtel 1977). Recently the NA neurons in the locus coeruleus (LC) which innervates almost the entire brain and spinal cord (Ungerstedt 1971, Lindvall & Björklund 1974, Nisgren & Olsson 1977) were found to be excited by microiontophoretically applied acetylcholine (ACh) (Bird & Kuhar 1977). Thus, the present study was undertaken to characterize the effect of nicotine on cholinergic receptor within the LC by utilizing single cell recording techniques and microiontophoretic application of various cholinergic agonists and antagonists onto LC noradrenergic neurons.

### METHODS

Male Sprague-Dawley rats (230–280 g) were used. The electrophysiological experiments were performed even-

tually as has been described previously (Svensson et al 1979, Svensson & Ulfhake 1978). The animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and mounted in stereotaxic apparatus. Additional injections were given as needed. Although general anesthetics seem to block excitatory muscarinic actions of ACh on cortical neurons (Catchlove et al 1972) this antagonism is not seen after chloralhydrate  $\alpha$ -g on hippocampal pyramidal cells (Bird & Aghajanian 1975) and also in the present study excitatory muscarinic effects of ACh were readily obtained. A 3 mm burr hole was drilled with its centre located 1.1 mm lateral to the midline and 1.1 mm posterior to lambda. For single-barrel experiments a micropipette with tip diameter of 1 µm filled with 0.1 M NaCl saturated with fast-green (impedance in vitro 3–7 MΩ measured at 135 Hz) was lowered with hydraulic microdrive into the brain. The electrode potentials were passed through high input-impedance amplifier and filters. Each spike was discriminated and fed into an integrator being reset every 10 or 1 s and finally displayed on an oscilloscope, an audiometer and an oscillographic recorder. Drugs are administered into tail vein. The body temperature of the animals was kept at 36–37°C. Only one cell was recorded in each animal. In some experiments microiontophoretic techniques were used utilizing cannulae eventually the same as previously described (Salmonague & Wright 1967) except that solid state design was chosen. Micropipettes with tip diameter of 4–5 µm were sealed with few strands of fibreglass inside prior to pulling to facilitate direct filling of the tips by capillary action (Tavakoli et al 1968). The central barrel was filled with 2 M CaCl<sub>2</sub> solution saturated with fast green as above and as used for recording action potentials. One of the side barrels contained 4 M CaCl<sub>2</sub> solution for automatic current balancing and the other

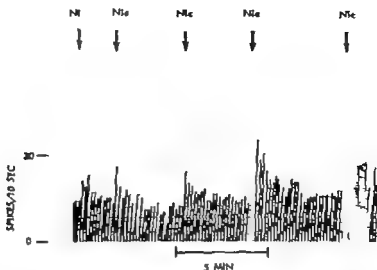


Fig. 1 Typical record showing the stimulation by nicotine (Nic: 10+20+20+50+50  $\mu\text{g/kg}$ ) administered of the firing rate of a noradrenergic neuron in the locus coeruleus in the rat brain. The effect is almost instantaneous, of short duration and dose-dependent.

contained drug solutions as listed below. The drug barrels contained 0.05 M or 0.2 M acetylcholine chloride, pH 6.2 (Sigma); 0.02 M, 0.1 M or 0.2 M nicotine bitartrate, pH 4.0; 0.2 M betanecol chloride, pH 3.5 (Sigma); 0.02 M L-glutamic acid in 0.2 M NaCl, pH 8.6 (B.D.H.) and/or 0.1 M scopolamine hydrochloride, pH 4.7 (ICN). The impedances were typically *in vitro* 2.5–5 M $\Omega$  to the central barrel, 20–100 M $\Omega$  in drug barrels. A retaining current of 10 nA was maintained between ejections, which were performed frequently to avoid dead-time artefacts.

A tentative LC neuron was localized utilizing several criteria (Korf et al. 1974) including its localization just rostral to the large cells of the mesencephalic nucleus of the Vth nerve, which typically respond to gentle movement of the jaw by bursts of activity. Furthermore, the LC cell are located just below the 4th ventricle, a zone of electrical silence and the spikes have a typical positive-negative wave form, often with a notch on the ascending limb. The characteristic rhythm and frequency (1–5 *imp/s*) and the typical response to noxious stimuli by a burst in activity followed by a quiescent interval (for details see Cedarbaum & Aghajanian 1978) was also observed. The final recording site was marked at the end of each experiment by iontophoretic ejection of fast-green. The rats were then perfused through the heart with 10% formaldehyde and serial 30  $\mu\text{m}$  frozen sections of the brain were cut, mounted and stained with cresyl violet and counterstained with neutral red. Only units within the LC were included in this study.

## RESULTS

When administered *i.v.* in small doses (10–50  $\mu\text{g/kg}$ ) nicotine caused a dose-dependent shortlasting in-

crease in the firing rate of the NA neurons in the LC (Fig. 1). Also physostigmine consistently produced increased firing of the LC-neurons and this effect was obtained even after pretreatment with methylscopolamine. In contrast, scopolamine rapidly antagonized the physostigmine-induced LC activation (Fig. 2). The microiontophoretic experiments revealed that nicotine, in contrast to ACh and the muscarinic agonist, betanecol (Fig. 3) did not activate the LC-cells, when tested in the various concentrations and at different ejection currents (0–50 nA). These experiments also showed that the muscarinic antagonist, scopolamine totally blocked the ACh-induced excitation of LC neurons. This blocking effect was obtained in a dose that did not at all inhibit the glutamate-induced excitation of these cells (Fig. 4). Other presumed nicotinic antagonists [(+)-tubocurarine, hexamethonium or gallamine] did not block the ACh excitation (data not shown).

## DISCUSSION

The present experiments show that nicotine in relatively small doses (10–50  $\mu\text{g/kg}$  *i.v.*) causes a dose-dependent shortlasting activation of NA-neurons in the LC. The short duration is in accordance with the very rapid accumulation in and disappearance from brain tissue previously reported (Schmitterlöw & Hansson 1965). The doses of nicotine

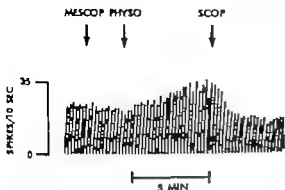


Fig 2 Pretreatment with the peripherally but not centrally active muscarinic receptor blocking agent methylscopolamine (MESCOPI 1 mg/kg i.v.) did not prevent the stimulation of the impulse activity of NA neuron in the LC by threshold dose of physostigmine (PHYSO 40 µg/kg i.v.). This effect was, however readily antagonized by the centrally active antagonist scopolamine (SCOP 0.25 mg/kg i.v.)

equivalent in those which produce cortical activation and behavioural arousal in animals (see Hall 1970). Recent studies have implicated the LC in maintenance of wakefulness and arousal reactions (Fuxe et al 1970; Foot & Bloom 1978; see Redmond 1977) as well as in modulation of autonomic e.g. cardiovascular function (see Amaral & Sinnamon 1977). Thus, electrical stimulation of the LC produced increased apprehensiveness, unrest and anxiety reactions in monkeys (Redmond et al 1976) as well as a pressor response in rats and cats (Przuntek & Philippu 1973; Ward & Gunn 1976). Consequently the stimulation by nicotine of LC neurons may form part of the underlying mechanism for such reactions in man following nicotine administration although the hypertensive response probably mainly is related to facilitated ganglionic transmission (Gebber 1969). The duration of the stimulatory effect of nicotine on brain NA-neurons corresponds to that of the hypertensive response obtained by the same doses (Armstrong et al 1968; Matsuoka & Domloof 1973). The LC stimulation is however probably not secondary to this response since acutely increased blood pressure e.g. obtained by intravenous injection of NA is not accompanied by increased LC cell firing rate (Engberg & Svensson unpublished observations).

The present data clearly suggest that the nicotine-induced LC stimulation is not directly mediated by cholinergic nicotinic receptors on the

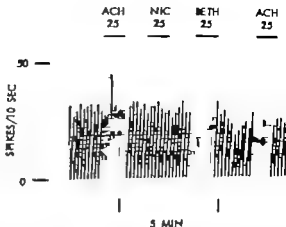


Fig 3 Integrated rate histogram showing the stimulation by microiontophoretically applied acetylcholine (ACh) and bethanechol (BETH) of the firing rate of a NA-neuron in the LC. In contrast, nicotine (NIC) did not cause any excitation. The drugs were applied at 45 nA for 1 min ejection periods (horizontal bars)

NA-neurons. This is so since both the LC stimulation by physostigmine and that of ACh when microiontophoretically applied, was blocked by scopolamine but not by the presumed nicotinic antagonists. The lack of antagonism by methylscopolamine indicates that peripheral muscarinic effects of physostigmine are of little significance for its LC stimulating action. Furthermore the muscarinic agonist bethanechol caused activation

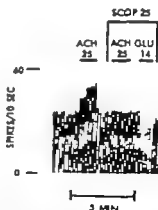


Fig 4 The ACh-induced increase in firing rate of NA cells in the LC totally blocked by the simultaneous microiontophoretic application of scopolamine (SCOP 25 nA). However the probably non-specific activation of the NA neuron by application of glutamate (GLU 10 nA) was unaffected by the muscarinic antagonist.

of LC neurons in contrast to nicotine when microiontophoretically applied. Thus the cholinergic excitatory receptor in the LC is of muscarinic character. This view is supported by the specificity of the ACh antagonistic action of scopolamine which did not block the NA-cell excitation by glutamate—a noncholinergic generally excitant amino acid (see Krnjević 1974).

In conclusion nicotine in small doses causes a probably indirect activation of NA neurons in the LC which are equipped with excitatory muscarinic cholinergic receptors.

This study was supported by the Swedish Medical Research Council (proj. no. 4747) and Swedish Tobacco Co. The excellent technical assistance of Mrs Birgitta Andersson is gratefully acknowledged.

## REFERENCES

- ANDÉN N E. & WACHTEL, H. 1977 Increase in turnover of brain dopamine by stimulation of muscarinic receptors outside the dopamine nerve terminals. *J Pharm Pharmacol* 29: 435-437.
- ARMITAGE, A K. HALL, G H & MORRISON C F. 1968 Pharmacological basis for the tobacco smoking habit. *Nature* 217: 331-334.
- BIRD S & AGHAJANIAN O K. 1975 Denervation supersensitivity in the cholinergic septo-hippocampal pathway: a microiontophoretic study. *Brain Res* 335: 370.
- BIRD S & KUCHAR, M J. 1977 Ionophoretic application of opiates to the locus coeruleus. *Brain Res* 122: 523-533.
- CATCHLOVE R F. KRNEVIĆ, K. & MARETIĆ II. 1972. Similarity between effects of general anesthetics and dimethylphenol on cortical neurones. *Can J Physiol Pharmacol* 50: 1111-1114.
- CEDARBAUM J M. & AGHAJANIAN O K. 1978. Activation of locus coeruleus neurons by peripheral stimuli. Modulation by a collateral inhibitory mechanism. *Life Sci* 23: 1383-1392.
- CORRODI H, FUXE K, HAMMER W, SJÖQVIST F & UNGERSTEDT U. 1967 Opiate and central monoamine neurons. *Life Sci* 6: 2557-2566.
- FOOT S & BLOOM F E. 1978 Activity of locus coeruleus neurons in the unanesthetized squirrel monkey. Abstract in Catecholamines: Basic and Clinical Frontiers, 4th Int. Catecholamine Symposium 17-22 Sept. 1978. Pacific Grove, California, p. 49.
- FUXE K, HOKFELT T & UNGERSTEDT U. 1970 Morphological and functional aspect of central monoamine neurons. *Int Rev Neurobiol* 13: 93-176.
- GEBBER, II. L. 1969 Neurogenic basis for the rise in blood pressure evoked by nicotine in the cat. *J Pharmacol exp Ther* 166: 235-263.
- HALL, G H. 1970 Effects of nicotine and tobacco smoke on the electrical activity of the cerebral cortex and olfactory bulb. *Br J Pharmacol* 38: 270-286.
- HALL, G H & TURNER, D M. 1972. Effect of nicotine on the release of <sup>3</sup>H-noradrenaline from the hypothalamus. *Biochem Pharmacol* 1: 1829-1838.
- KORF J, BUNNEY B S. & AGHAJANIAN O K. 1974 Noradrenergic neurons: Morphine inhibition of spontaneous activity. *Eur J Pharmacol* 25: 165-169.
- KRNEVIĆ, K. 1974 Chemical nature of synaptic transmission in vertebrates. *Physiol Rev* 54: 418-540.
- LINDVALL, O & BJÖRKLUND A. 1974 The organization of the ascending catecholamine neuron system in the rat brain as revealed by the glyoxylic acid fluorescence method. *Acta Physiol Scand Suppl.* 412: 1-48.
- MATSUOKA, I & DOMINO E F. 1972. Cholinergic modulation of single lateral geniculate neurons in the cat. *Neuropharmacol* 11: 241-251.
- NYGREN L. & OLSON L. 1977 A new major projection from locus coeruleus, the main source of noradrenergic nerve terminals in the ventral and dorsal columns of the spinal cord. *Brain Res* 132: 85-93.
- PRZUNTEK, H & PHILIPPU A. 1973 Reduced pressor response to stimulation of the locus coeruleus after lesion of the posterior hypothalamus. *Naunyn-Schmiedeberg Arch Pharmacol* 276: 119-122.
- REDMOND D E, Jr. 1977 Alterations in the function of the nucleus locus coeruleus, a possible model for studies of anxiety. In *Animal Models in Psychiatry and Neurology* (Eds. I. Haulin and E. Usdin.) Oxford, New York: Pergamon Press 1977 pp. 293-305.
- REDMOND D E, Jr. HUANG Y H, SNYDER D R. & MAAS J W. 1976. Behavioral effects of stimulation of the nucleus locus coeruleus in the stump-tailed monkey *Macaca arcoides*. *Brain Res* 116: 503-510.
- SALMOURAGHI G C. & WEIGHT F. 1967 Micro-methods in neuropharmacology: an approach to the study of anesthetics. *Anesthesiology* 28: 54-64.
- SCHMITTLERLOW C G & HANSSON E. 1965 The distribution of <sup>14</sup>C-nicotine. In: *Tobacco Alkaloids and Related Compounds* (ed. U S von Euler) Pergamon Press, N.Y., pp. 75-86.
- SVENSSON T H, BUNNEY B S & AGHAJANIAN O K. 1975 Inhibition of both noradrenergic and serotonergic neurons in brain by the  $\alpha$ -adrenergic agonist clonidine. *Brain Res* 92: 291-306.
- SVENSSON T H & USIDIN T. 1978 Feed-back inhibition of brain noradrenergic neurons by tricyclic  $\alpha$ -receptor mediation after acute and chronic treatment. *Science* 202: 1069-1091.
- TASAKI K, TSUKUHARA U, ITO S, WAYNER, M J & YU W Y. 1968. A simple, direct and rapid method for filling microelectrodes. *Physiol Behav* 3: 1009-1010.
- UNGERSTEDT U. 1971 Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol Scand Suppl* 367: 1-48.
- WARD D G & GUNN C G. 1976. Locus coeruleus complex: elicitation of a pressor response and brain stem region necessary for its occurrence. *Brain Res* 107: 401-406.
- WESTFALL, T C. 1974 Effect of nicotine and other drugs on the release of H-norepinephrine and H-dopamine from rat brain slices. *Neuropharmacol* 13: 639-700.

## Effect of chronically administered nicotine on axonal transport of dopamine- $\beta$ -hydroxylase in peripheral adrenergic neurons and on blood pressure and heart rate in the rat

JAN HÄGGENDAL and MATTS HENNING

Department of Pharmacology University of Göteborg, Göteborg, Sweden

**ABSTRACT** Nicotine was given to rats of about 200 g b.wt. in the drinking water for up to 2 months. Different doses of nicotine, from 25 mg to 100 mg per litre water, were tested with respect to their reducing effect on the normal increase in body weight. No effect was found on the normal accumulation of dopamine- $\beta$ -hydroxylase (DBH) proximal to ligation of peripheral adrenergic neurons. This indicated normal turnover of axonal storage granules. This was interpreted as nicotine being without effects on the activity of the adrenergic neurons. In agreement with this lack of effect no changes of blood pressure or heart rate were found in rats receiving 50 mg nicotine per litre of drinking water.

**Key words** Nicotine chronic treatment, sympathetic activity, dopamine- $\beta$ -hydroxylase transport, blood pressure, heart rate.

Effects of nicotine on the peripheral autonomic system have been studied for more than one hundred years (for review see Trendelenburg 1965). The pharmacological properties of nicotine are very complex with stimulating as well as inhibiting actions on both the peripheral nervous system and the central nervous system. Much attention has been paid to the stimulating effect of nicotine on the sympathetic ganglia and the adrenal medulla. A directly stimulating effect of nicotine on the peripheral adrenergic nerve terminals has also been reported (e.g. Su & Bevan 1970, Westfall & Brasted 1972, Nedergaard & Schrold 1977). Nicotine may thus by acting at different levels in the adrenergic neuron increase the activity of the neuron and cause increased release of the transmitter noradrenaline (NA).

Such an increased sympathetic activity has been discussed to cause stimulation of the cardiovascular system giving increased blood pressure and increased heart rate (see e.g. Völle & Koelle 1975). In most experiments, however, nicotine has been given in a single dose, while a smoker is exposed to chronic treatment with nicotine.

Studies on the long-term effect of nicotine on the

activity of the adrenergic neurons and on the cardiovascular system are rather few and the results are partly contradictory (compare e.g. the results of Westfall 1970 with those of Wenzel & Azmet 1970).

The activity in sympathetic adrenergic neurons has been studied indirectly by measuring the amount of dopamine- $\beta$ -hydroxylase (DBH) accumulated per time unit above a crush or a constriction of adrenergic axons. The background for this approach was as follows. The transmitter NA is stored in amine storage granules (Euler & Höllarp 1966). The NA has to be stored in granules in order to be released from the nerve terminals during physiological nerve activity (cf. Andén, Carlsson & Häggendal 1969). The granules are complex structures containing not only NA but also proteins e.g. DBH and during nerve activity NA and also to some extent DBH will be released (cf. Geffen & Lott 1971). The granules will thus be worn-out and have to be replaced by newly formed granules. In all probability the new granules are formed in the cell bodies and transported down to the nerve terminals (cf. Dahlström 1971, Häggendal & Dahlström 1971). The amounts of granular components, such as DBH or NA, accumulating above a constriction of the



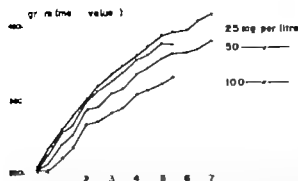


Fig. 1 Body weights of rats with or without nicotine in the drinking water. The values indicated are mean values calculated from groups of 5 animals. 2-5 groups have been used for every point. Nicotine was given as bitartrate and the doses indicated correspond to pure nicotine.

axons will reflect the production of new granules. This production is increased at increased nerve activity and decreased at decreased nerve activity in agreement with "trans-synaptic induction" (Thoenen, Mueller & Axelrod 1969; Molinoff & Brumojin & Axelrod 1971).

The nicotine was given in the drinking water in this study. This may be a way for chronic administration of nicotine that comes closer to that used by a smoker than injections of nicotine do, even when the injections are given several times a day.

Since nicotine by changing the sympathetic activity may influence blood pressure and heart rate, these parameters have been followed. By recording directly from implanted catheters the use of anaesthesia or handling of the animals during the registration could be avoided.

## METHODS

Male Sprague-Dawley rats with an initial body weight of about 200 g were used. They were kept in groups of 5 animals (at 23°C) with food and water (with or without nicotine added in different concentrations) available *ad libitum*. The weight of the different groups was measured twice a week. Food intake and water consumption were measured at intervals.

At different time intervals during the nicotine treatment sciatic nerve crushes were performed bilaterally under ether anaesthesia *s.c.* Lubrinski (1959). 6 or 9 h later the rats were killed and the nerves dissected out and divided to 5 or 10 mm pieces proximal and distal to the crush. The DBH content in the different pieces of the nerves was determined according to Nagatsu & Udenfriend (1972) as DBH activity.

In some rats, ligations were also performed around the

perihilar tissue of the salivary glands (gl. submaxillaris and gl. sublingualis) 5 h later. The tissue proximal to the ligation was dissected out in a 4 mm long portion. This tissue, containing ligated adrenergic axons, was also assayed for its content of DBH.

For recordings of arterial blood pressure and heart rate aortic catheters were implanted under pentobarbital anaesthesia as previously described (Henning 1969). Recordings were made in conscious unrestrained animals no less than 24 h after surgery. Heart rate was triggered from the pressure signal. The values given represent the average of two 30 min recordings in one day.

## RESULTS AND DISCUSSION

The effect of nicotine (added in different doses to the drinking water as nicotine base or nicotine bitartrate) on the increase in body weight was followed. Nicotine treatment resulted in a dose dependent reduction of the normal weight increase (Fig. 1). No difference was found in the effect between nicotine base and nicotine bitartrate when the same doses with respect to pure nicotine were used.

After 2 weeks of treatment with 50 mg of nicotine per litre of the drinking water, the daily consumption of water was found to be about constant around 28 ml per day. This corresponds to an intake per day of about 1.4 mg of nicotine. The body weight increased from about 280 g after 2 weeks of treatment to about 360 g after 6 weeks. The daily consumption of nicotine will thus be between 5 mg per kg after week 2 and 3.9 mg per kg after week 6. The one pack-a-day cigarette equivalent has been discussed to be 1.4 mg per kg and day.

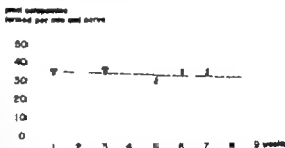


Fig. 2 Dopamine- $\beta$ -hydroxylase (DBH) accumulated per hour proximal to crush of the sciatic nerve of rats with or without nicotine in the drinking water. DBH is expressed as DBH activity in  $\mu$ mol octopamine formed per minute and nerve piece. (O) indicates no nicotine in the drinking water, (●) indicates 50 mg nicotine base per litre of water, (□) and (Δ) indicates nicotine bitartrate corresponding to 50 and 100 mg pure nicotine per litre, respectively. Every point is the mean value, generally of 4 to 5 estimations.

Table 1 Mean arterial blood pressure (mmHg) and heart rate (beats/min) during two consecutive days in conscious rats with or without nicotine in the drinking water

The values are means with S.E. and number of rats in brackets

	Heart rate		Blood pressure	
	Day 1	Day 2	Day 1	Day 2
Controls (no nicotine)	337 8.2 (11)	323 16.7 (5)	131 4.4 (11)	125 5.7 (5)
Nicotine (50 mg nicotine base per litre drinking water)	321 7.9 (14)	320 10.3 (6)	13 3.1 (14)	131 6.5 (6)

(Wenzel & Azmeh 1970). It is hard to compare man's intake of nicotine by smoking with intake of nicotine in the drinking water in these experiments but since we are dealing with different concentrations in the water from 2.5 mg to 100 mg per litre some of the concentrations may be relatively relevant to the nicotine consumption of a smoker.

The water intake was higher in controls than during nicotine treatment. After 3 weeks the consumption was constant, about 45 ml per day in controls. Based on circulatory data, blood pressure and heart rate the rats given 50 mg nicotine per litre water do not appear to have been dehydrated to any considerable extent. It is of interest to note that a marked reduction in body weight as compared to controls has been reported in rats receiving parenteral nicotine (0.4 or 0.8 mg per kg) 3 times a day (Schechter & Cook 1976). Furthermore it was observed in this study that the food intake was about the same in the two groups.

The effect of different doses of nicotine on the accumulation of DBH above a constriction of the sciatic nerve is shown in Fig. 2. As can be seen there was no clearcut effect of nicotine as compared to the controls. Moreover there was no effect of nicotine on the accumulation of DBH above a constriction of the adrenergic axons to the salivary glands (data not shown).

This lack of action of nicotine on the accumulation of DBH above a constriction of adrenergic axons strongly indicates that the production of new axons was uninfluenced upon by nicotine in the doses used. It appears therefore unlikely that the activity of the adrenergic neurons was changed by

the nicotine. Several theoretical possibilities for such a lack of effect may be discussed. However it may be of more direct interest to study the effect of nicotine on the circulatory system.

Table 1 shows that after 30 days of treatment with 50 mg nicotine per litre in the drinking water there was no effect of nicotine either on blood pressure or on heart rate. One explanation for this lack of effect as compared to other investigators may be differences in the methods used for the registration of the circulatory parameters.

When the chronic treatment with nicotine was terminated and pure water was given some of the groups showed signs of abstinence both with respect to DBH-accumulation and circulatory parameters. These results will be presented elsewhere.

The results of this study show that even when nicotine is given in the drinking water in doses which retard the normal increase of body weight there were no signs of increased activity of peripheral adrenergic neurons nor were any changes in blood pressure or heart rate observed. This seems to be in agreement with the clinical observations that smoking and hypertension are not imperatively correlated (for ref. see Wenzel & Azmeh 1970).

This study was supported by Svenska Tobaks AB\* and The Swedish Medical Research Council (project Nos. 166 and 2863).

## REFERENCES

- ANDÉN N. E., CARLSSON A. & HÄGGENDAL, J. 1969. Adrenergic mechanisms. *Ann. Rev. Pharmacol.* 9: 119-134.
- DAHLSTRÖM A. 1971. Azoplasma transport with particular respect to adrenergic neurons. *Phil. Trans. Roy. Soc. B* 261: 325-358.
- EULER, U. S. & HILLARP, N. Å. 1946. Evidence for the presence of noradrenaline in submicroscopic structure of adrenergic axons. *Nature (Lond.)* 177: 44-45.
- GEFFEN, L. B. & LIVETT, B. J. 1971. Synaptic vesicles in sympathetic neurons. *Physiol. Rev.* 51: 98-157.
- HÄGGENDAL, J. & DAHLSTRÖM A. 1971. The functional role of the amine storage granules of the sympathetic-adrenal system. In *Mem. Soc. Endocrinol.* (ed. H. Heller and K. Lederis), 19: 651-669.
- HENNING, M. 1969. Studies on the mode of action of  $\alpha$ -methyltyrosine. *Acta Physiol. Scand. Suppl.* 322.
- LUBINSKA, L. 1959. Region of transition between peripheral and regenerating parts of myelinated nerve fibres. *J. Comp. Neurol.* 113: 315-335.
- MOLINOFF, P. B., BRIMOUIN, S. & AXELROD, J. 1971. Trans-synaptic induction of dopamine (3,4-di-

- hydroxyphenethylamine)  $\beta$ -hydroxylase in adrenergic tissues of the rat. *Biochem J* 123 (4): 32-33P
- NAGATSU T & UDENFRIEND S 1972. Photometric assay of dopamine- $\beta$ -hydroxylase activity in human blood. *Clin Chem* 18: 980-983
- NEDERGAARD O A. & SCHROLD J 1977. The mechanism of action of nicotine on vascular adrenergic neuroeffector transmission. *Eur J Pharmacol* 42: 315-329
- SCHECHTER, M ■ & COOK P O 1976. Nicotine-induced weight loss in rats without an effect on appetite. *Eur J Pharmacol* 38: 63-69
- SU C & BEVAN J A. 1970. Blockade of the nicotine-induced norepinephrine release by cocaine, phenoxybenzamine and desipramine. *J Pharmacol Exp Ther* 175: 533-540
- THOENEN H, MUELLER, R. A & AXELROD J 1969. Trans-synaptic induction of adrenal tyrosine hydroxylase. *J Pharmacol Exp Ther* 169: 249-254
- TRENDELENBURG U 1965. Nicotine and the peripheral autonomic nervous system. In: Tobacco alkaloids and related compounds (ed. U. S. v. Euler). Pergamon Press, New York, 167-177
- VOLLE, R. L. & KOELLE, G. B. 1975. Gaseous stimulating and blocking agents. In: The pharmacological basis of therapeutics (ed. L. S. Goodman and A. Gilman). The Macmillan Company. Fifth edition. Chapter 27: 565-574
- WENZEL, D. G. & AZMEH ■ 1970. Chronically administered nicotine and the blood pressure of normotensive and renal hypertensive rats. *Arch Int Pharmacodyn* 187: 367-376
- WESTFALL, T. C. 1970. Influence of chronic nicotine administration on blood pressure and heart norepinephrine turnover. *Eur J Pharmacol* 10: 19-4
- WESTFALL, T. C. & BRASTED M. 1972. The mechanism of action of nicotine on adrenergic neurons in the perfused guinea-pig heart. *J Pharmacol Exp Ther* 182: 409-418.

## Cholinergic inhibition of sympathetic vasoconstrictor tone in the cerebrovascular bed mediated by nicotinic type receptors

CH OWMAN, P AUBINEAU, L EDVINSSON and R SERCOMBE

Department of Histiology University of Lund, Lund, Sweden and Laboratoire de Physiologie et Physiopathologie Cérébrovasculaire, Département de Neurophysiologie de la Faculté de Médecine Lariboisière Saint Louis, Université de Paris VII, France

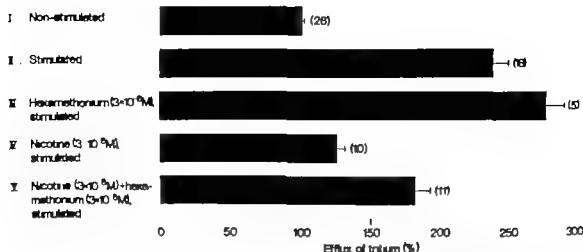
**ABSTRACT** The cholinergic influence on the cerebrovascular bed was studied in cats and rabbits using a combination of *in vitro* and *in vivo* approaches. Isolated pial arteries were superfused with  $^3\text{H}$ -norepinephrine and the efflux of tritium was studied under various conditions of transmural electrical stimulation of the perivascular sympathetic nerves. Field stimulation increased the radioactivity in the efflux in a frequency-dependent manner. The amount of tritium overflow (of which approximately 70% represents liberated radioactive norepinephrine) from the nerves during stimulation was enhanced by hexamethonium, the effect being inhibited by nicotine or acetylcholine. The action of nicotine and acetylcholine was counteracted by hexamethonium (but not by atropine). Carbachol infusion under *in vivo* conditions increased blood flow in the caudate nucleus as measured by thermoclearance. This effect was inhibited by atropine. Postganglionic stimulation of the cervical sympathetic chain above the superior cervical ganglion produced, in itself, a reduced local cerebral blood flow. The response (but not the flow reduction obtained by exogenous norepinephrine) was diminished during infusion with carbachol. The inhibition amounting more than 90% of the sympathetic nerve action on CN blood flow was not affected by atropine. It is concluded that (a) there are direct cholinergic dilator mechanisms in the cerebrovascular bed mediated by muscarinic-type of cholinergic receptors in the vascular smooth musculature and (b) the perivascular adrenergic nerve terminal possesses nicotinic-type of cholinergic receptors mediating an inhibition of the local norepinephrine release, probably through an action by the cholinergic nerve terminals, which run parallel to and in close association with the adrenergic axons separated only by 25 nm distance.

On the basis of cholinesterase histochemistry following appropriate inhibition of pseudocholinesterase it has been shown that the pial vessels are supplied by numerous cholinergic nerves which run along with the adrenergic axons in the same strands of the perivascular autonomic plexus (Edvinsson et al 1977). Electron microscopy has demonstrated that both systems of nerve terminals form close contacts with the vascular smooth muscle cell in a manner suggesting a functioning innervation (Nielsen et al 1971). Both  $\alpha$ -adrenergic and  $\beta$ -adrenergic receptors have been identified and characterized in pharmacological experiments (Edvinsson & Owman 1974). The findings that pial vessels respond to acetylcholine whose effect is blocked by atropine (Edvinsson et al 1977, Ow-

man et al 1978) suggest the presence also of cholinergic receptors in the vascular wall. The close association between apposing adrenergic and cholinergic axon terminals (Nielsen et al 1971, Edvinsson et al 1972) has indicated that the situation is complex and probably includes a terminal interaction between the two nerve systems (Edvinsson et al 1977) whereby liberated acetylcholine influences not only the vascular smooth musculature but also the adjacent adrenergic nerves at the sites of apposition.

### METHODS

Pial artery preparations were obtained from the brain of adult cat of either sex weighing



**Fig. 1** Measurements of tritium overflow in pial vessels. Pronounced increase during electrical field stimulation (II) in comparison with nonstimulated controls ( $p < 0.001$ ). The increased efflux during stimulation is enhanced by hexamethonium (III  $p < 0.05$ ) and inhibited by nicotine (IV  $p < 0.05$  in comparison with nonstimulated controls I). The nicotine effect seen during transneuronal stimulation is counteracted by hexamethonium (V  $P < 0.01$  in comparison with IV). The mean amount of radioactivity in three 0.5-ml samples taken every minute before stimulation is set as 100%. Number of experiments within parentheses.

Sympathectomy was carried out under sodium pentobarbital (30 mg/kg, i.p.) anaesthesia by excision of the superior cervical ganglia at least 1 week prior to the experiments. The animals were killed by bleeding under the same anaesthesia, the brain was removed and the pial vessels were immediately dissected out and placed in aerated Krebs-Ringer buffer solution of the following composition (mM): NaCl 118, KCl 4.5,  $\text{MgSO}_4$  7,  $\text{H}_2\text{O}$  1.0,  $\text{KH}_2\text{PO}_4$  1.0,  $\text{NaHCO}_3$  25,  $\text{CaCl}_2$  2,  $\text{H}_2\text{O}$  2.5, glucose 6.0. The tissue

for studies of the norepinephrine release during oral electrical stimulation consisted of 2–3 pieces of al arteries up to 10 mm long which were pre-incubated for 20 min at  $37^\circ\text{C}$  in Krebs-Ringer buffer solution (aerated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ) containing  $10^{-6}$  M of  $^3\text{H}$ -norepinephrine (labelled at the  $\beta$ -carbon atom 30 mCi/mg). Either intact or sympathetically denervated vessels were used. After pre-incubation they were transferred to a superfusion chamber equipped with platinum electrodes for electrical field stimulation (Edvinsson et al. 1977) according to the technique described by Farneth (1971). The chamber was perfused with pre-warmed ( $37.5^\circ\text{C}$ ) Krebs-Ringer solution and the efflux collected (one 0.5 ml sample every minute) as liquid scintillation vials. After addition of 10 ml Instagel (Packard) counting of radioactivity was performed in Packard Tri-Carb liquid scintillator.

Following incubation in the presence of  $^3\text{H}$ -norepinephrine, the pial vascular preparations were transferred to small chamber and superfused with buffer and the radioactivity of the overflow was measured in the efflux before and during electrical field stimulation by 12 V (measured by an oscilloscope between the electrodes in the chamber), 1 ms pulse duration and 10 Hz frequency.

The basal level of tritium overflow was determined as the mean amount obtained during 3 min period before stimulation, and was set as 100%. The efflux was then

stimulated electrically by Grass Model S44 stimulator and the efflux collected during a further period of  $3 \times 1$  min for determination of a new mean level of radioactivity. Three control samples were again collected without stimulation. The antagonists were added to the superfusate 15 min before (and remained present during) next stimulation period. The agonists were similarly added 5 min before. The viability of the system was checked by a final 3 min period of electrical stimulation in the absence of drugs, followed by a 3 min control period.

#### Experiments in

Blood flow was measured in the caudate nucleus (CN) by heat clearance (Seylaz 1965; Seylaz et al. 1973; Sercombe et al. 1975). The thermistor probes were implanted chronically and the animals were used 10–14 days later possible inflammation or edema had then been resorbed.  $\text{PaO}_2$  and  $\text{PaCO}_2$  were measured continuously by mass spectrometry (Seylaz et al. 1974). Arterial blood pressure (B.P.) was measured in the aorta via conventional pressure transducer.

Thirteen rabbits of either sex, weighing about 3 kg, were anesthetized by diazepam-pentobarbital. After tracheal cannulation careful dissection of the sympathetic trunk and superior cervical ganglion was made. Pairs of stimulating electrodes were placed on the nerve trunk and the distal part of the ganglion, while a hypodermic needle, 0.3 mm d. and connected to 3  $\mu\text{l}$  Hamilton syringe, was implanted into the proximal part of the ganglion. Another such needle was implanted in the common carotid artery. A Teflon catheter was introduced into the aorta via the femoral artery; this was used to guide into place the mass spectrometer cannula, and also to measure the blood pressure and take blood samples for analysis by polarography.

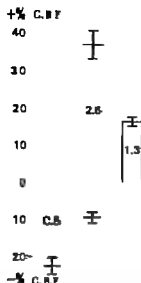


Fig. 2 Diagram summarizing cholinergic effects on local cerebral blood flow (C.B.F.) measured in the CN. Open bars: Mean increase ( $\pm$ S.E.) in CN blood flow during intracarotid infusion with 2.5 or 1.3  $\mu$ g/kg/min of carbachol. Supplied bars: Mean reduction ( $\pm$ S.E.) in CN blood flow during postganglionic cervical sympathetic nerve stimulation (C.S.), alone or during the intracarotid infusion of 2.5  $\mu$ g/kg/min of carbachol.

## RESULTS

### Experiments *in vitro*

In non-sympathectomized vessels the spontaneous rate of tritium overflow during a 3-min sampling period corresponded to  $2836 \pm 456$  cpm (mean  $\pm$  S.E.). Electrical field stimulation significantly increased the radioactivity in the efflux, the effect being more pronounced with increasing frequency tested at 3, 6 and 10 Hz. After cessation of stimulation the amount of radioactivity returned to a level slightly below that recorded before stimulation (cf. Farnebo 1971). The increase of radioactivity in the efflux (at a stimulation frequency of 10 Hz) was abolished by the presence of  $6 \times 10^{-6}$  M bretylium or  $10^{-4}$  M guanethidine in the buffer solution (Edvinsson et al. 1977).

In the presence of hexamethonium, the electrical field induced overflow of tritium was enhanced (Fig. 1). The effect of electrical field stimulation was on the other hand markedly inhibited by  $3 \times 10^{-6}$  M acetylcholine or  $3 \times 10^{-6}$  M nicotine (Fig. 1). The effect of either acetylcholine or nicotine could be counteracted by  $3 \times 10^{-6}$  M hexamethonium (Fig. 1) but not by atropine, tested in doses up to  $10^{-5}$  M.

(i.e. doses which had been shown to have marked effects on the motor response of the pial vessels to acetylcholine as mediated by the muscarinic receptors Edvinsson et al. 1977). The spontaneous efflux of tritium (from non-stimulated vessels) was not altered when nicotine was added to the perfusion solution in a concentration of  $3 \times 10^{-6}$  M.

Following sympathetic denervation of the pial vessels the radioactivity in the efflux was only  $292 \pm 86$  cpm and it was not altered by electrical field stimulation as tested at a frequency of 10 Hz (Edvinsson et al. 1977).

### Experiments *in vivo*

Before each test with carbachol the ganglionic nicotinic synapses were blocked by local injection of 0.5–2.0  $\mu$ l of hexamethonium solution thus avoiding possible pharmacological stimulation of postganglionic neurons. Total blockade of the transmission was verified by the suppression of the effects of preganglionic stimulation on the iris and on the CN blood flow.

The effect of postganglionic stimulation (after local ganglionic transmission blockade) on the CN blood flow was studied (see also Aubineau et al. 1977). A large number of identical stimulations were carried out before, during and after intracarotid infusion of 2.5  $\mu$ g/kg/min carbachol. During the control stimulations a fall in flow of  $22.0 \pm 2.5\%$  (mean  $\pm$  S.E.) was found (Fig. 2). A few min after the beginning of the carbachol infusion the same stimulation induced a fall only  $9 \pm 1.8\%$  (Fig. 2). This inhibition disappeared progressively until 5–10 min after the end of the carbachol infusion, where after the effects of stimulation were as before. The inhibition of sympathetic nerve action thus attained  $58 \pm 20\%$ . It was not diminished by doses of atropine which almost totally inhibited the flow rise induced by carbachol. The diminution of CN blood flow induced by norepinephrine injections were unchanged during carbachol perfusion and under the influence of atropine. It may be noted that carbachol appeared to potentiate the rise in flow following sympathetic stimulation, whether or not atropine was present.

## COMMENTS

Methodological questions regarding the validity of the superfusion technique to study sympathetic

nerve activity during electrical field stimulation have been discussed elsewhere (Edvinsson et al 1977).

The amount of increase in radioactivity recovered in the effluent during *in vitro* stimulation of the perivascular sympathetic nerves was significantly reduced by the presence of acetylcholine or nicotine the effect being counteracted by hexamethonium but not by atropine. Similarly the amount of fall in CN blood flow during sympathetic nerve stimulation *in vivo* was reduced by infusion of carbachol an effect that was not inhibited by atropine. The experiments suggest the presence of a nicotinic type of cholinergic receptors on the perivascular sympathetic nerves which was corroborated by the finding *in vitro* that pretreatment of the superfused brain vessels with hexamethonium enhanced the increment in tritium overflow during transmural stimulation. A further indication that the site of this inhibitory action of carbachol is on the adrenergic nerve was given by the fact that the vasoconstriction obtained by systemic administration of norepinephrine was not modified during the carbachol infusion. In view of the close axo-axonal relationship between the adrenergic and cholinergic nerve varicosities (Nielsen et al 1971, Edvinsson et al 1977) it is possible that the cholinergic nerve terminals can act synaptically on adjacent sympathetic nerves in the vessel wall. Since the adrenergic sympathetic nerves appear to be of vasoconstrictor nature (see Owman et al 1978) it follows that the cholinergic system besides its direct (postjunctional) vasodilatory action on muscarinic receptors in the vascular smooth muscle can promote vasodilatation also indirectly through an inhibition of the norepinephrine release via the nicotinic receptors present on the perivascular sympathetic fibres.

Supported by grants from the Swedish Medical and Natural Sciences Research Councils, the Swedish Tobacco Foundation and INSERM and CNRS (France).

## REFERENCES

- AUBINEAU P, SERCOMBE, R., EDVINSSON L. & OWMAN CH 1977 Cholinergic influence on the cerebrovascular bed mediated through actions on the vascular smooth musculature and on the perivascular sympathetic nerves. *In*: Neurogenic control of the brain circulation (ed. Ch. Owman and L. Edvinsson), pp. 331-342. Pergamon Press, Oxford.
- EDVINSSON L., FALCK, B. & OWMAN CH. 1977 Possibilities for a cholinergic action on smooth musculature and sympathetic axons in brain vessels mediated by muscarinic and nicotinic receptors. *J Pharmacol Exp Ther* 200: 117-126.
- EDVINSSON L., NIELSEN N. C., OWMAN CH. & SPORRONG B. Cholinergic mechanisms in pial vessels. *Histochemistry electron microscopy and pharmacology* *Z. Zellforsch* 134: 311-325 (1972).
- EDVINSSON L. & OWMAN CH. 1974 Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries *in vitro*. *Circulation Res* 35: 835-849.
- FARNEBO L.-O. 1971 On transmitter release evoked by field stimulation of monoamine neurons. P. A. Norstedt and Soner, Stockholm.
- NIELSEN K. C., OWMAN CH. & SPORRONG B. 1971 Ultrastructure of the autonomic innervation apparatus in the main pial arteries of rats and cats. *Brain Res* 77: 3-32.
- OWMAN CH., EDVINSSON L. & HARDEBO J. E. 1978 Pharmacological *in vitro* analysis of acetylcholine-mediated vasomotor functions in the intracranial and extracranial vascular beds. *Blood Vessels* 15: 128-147.
- SERCOMBE, R., AUBINEAU P., EDVINSSON L., MAMO H., OWMAN CH., PINARD E. & SEYLAZ, J. 1975 Neurogenic influence on local cerebral blood flow. Effect of catecholamines or sympathetic nerve stimulation as correlated with the sympathetic innervation. *Neurology* 25: 954-963.
- SEYLAZ, J. 1965 Développement d'une méthode de mesure instantanée localisée et semi-quantitative du débit sanguin *in vivo*. *Biophysik* 2: 330-339.
- SEYLAZ, J., AUBINEAU P. F., CORRÈZE, J. L. & MAMO H. 1973 Techniques for continuous measurement of local cerebral blood flow.  $PaO_2$ ,  $PaCO_2$  and blood pressure in the nonanesthetized animal. *Pflügers Arch* 340: 173-180.
- SEYLAZ, J., PINARD E., CORRÈZE, J. L., AUBINEAU P. F. & MAMO H. 1974 Quantitative continuous measurement of blood gas tensions by mass spectrometry. *J Appl Physiol* 37: 937-941.

## Effects of tobacco smoking on the blood temperature during exercise

TOMAS BRUNDIN

Department of Clinical Physiology Huddinge Hospital S-141 86 Huddinge Sweden

**ABSTRACT** By thermometry in mixed venous blood the thermal reactions to exercise programmes of different durations were studied in healthy volunteers before and after smoking. Pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ), arterio-venous oxygen difference (AVD) and heart rate (HR) were also measured. It was found that exercise-induced rise in blood temperature was considerably augmented after smoking. This effect was paralleled (and probably partly caused) by an increased heat production reflected by increased  $\dot{V}_{O_2}$ . During short-term exercise an accelerated rise in AVD might indicate that centripetal redistribution of the total blood flow could have contributed to the thermal response by a reduced skin blood flow. During long-term exercise cardiac output and heart rate increased and the stroke volume decreased in response to smoking.

In studies on the blood temperature in healthy men during exercise remarkably high temperature values were sometimes noted in habitual smokers. Inspired by this observation the present study was undertaken in order to find out whether tobacco smoking *per se* might interfere with the thermal adaptation to dynamic exercise.

By means of a technique developed for direct thermometry in mixed venous blood (Brundin 1975; Kilbom & Brundin 1976) the thermal response to exercise was measured in healthy individuals during standardized exercise programmes of different durations. The effect of tobacco smoking was studied by repeating the exercise programme after smoking. The results were compared with those from control groups who did not smoke between the exercise periods. The effects on pulmonary oxygen uptake, arterio-venous oxygen difference, cardiac output, stroke volume and heart rate were simultaneously studied.

### MATERIAL AND METHODS

16 healthy volunteers, 22-40 years of age, all habitual smokers, were studied. They came to the laboratory in the morning after light breakfast. They are not allowed to smoke for 12 h before the study. All the subjects were informed of the nature, purpose and possible risk in-

volved in the study before giving their voluntary consent to participate.

A teflon catheter (1.1 mm outer diameter) was inserted percutaneously under local anaesthesia into a brachial artery. A thermostat-equipped catheter (Swan-Ganz 93-118 F, Edwards Laboratories) was inserted into an antecubital vein and passed to the pulmonary artery under fluoroscopic control.

18 of the subjects, mixed venous blood temperature (PA-temp), arterio-venous oxygen difference (AVD) and heart rate (HR) were measured at rest and during two identical programmes of short-term exercise performed with 30-40 min intermission. During the pause 5 subjects had to smoke 1 cigarette before the second exercise period was started. 3 subjects served as controls and repeated the exercise programme without smoking.

Exercise was performed in the supine position on bicycle ergometer starting at work load of 20 W. The load was increased by 10 W each minute. The total exercise duration was 8 min and the final work load was 90 W. For none of the subjects the exercise was exhaustive or tiresome; the load being far below their maximum working capacity.

18 subjects, steady state exercise programme was used. Bicycle exercise was performed at constant work load of 40 W for 30 min. PA-temp, AVD, HR and pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ) were measured at rest at 17-20 min and at 27-30 min after the onset of exercise. At 20-25 min 5 of the subjects had to smoke 1 cigarette without interruption of the exercise before the final measurements. 3 of the subjects served as controls by performing the exercise programme without smoking.

The room temperature was kept around 23°C. The collected expiration air was analysed for oxygen and carbon



nerve activity during electrical field stimulation have been discussed elsewhere (Edvinsson et al 1977)

The amount of increase in radioactivity recovered in the effluent during *in vitro* stimulation of the perivascular sympathetic nerves was significantly reduced by the presence of acetylcholine or nicotine the effect being counteracted by hexamethonium but not by atropine. Similarly the amount of fall in CN blood flow during sympathetic nerve stimulation *in vivo* was reduced by infusion of carbachol an effect that was not inhibited by atropine. The experiments suggest the presence of a nicotinic type of cholinergic receptors on the perivascular sympathetic nerves which was corroborated by the finding *in vitro* that pretreatment of the superfused brain vessels with hexamethonium enhanced the increment in tritium over flow during transmural stimulation. A further indication that the site of this inhibitory action of carbachol is on the adrenergic nerve was given by the fact that the vasoconstriction obtained by systemic administration of norepinephrine was not modified during the carbachol infusion. In view of the close axo-axonal relationship between the adrenergic and cholinergic nerve varicosities (Nielsen et al 1971, Edvinsson et al 1972) it is possible that the cholinergic nerve terminals can act synaptically on adjacent sympathetic nerves in the vessel wall. Since the adrenergic sympathetic nerves appear to be of vasoconstrictor nature (see Owman et al 1978) it follows that the cholinergic system besides its direct (postjunctional) vasodilatory action on muscarinic receptors in the vascular smooth muscle can promote vasodilatation also indirectly through an inhibition of the norepinephrine release via the nicotinic receptors present on the perivascular sympathetic fibres.

Supported by grants from the Swedish Medical and Natural Sciences Research Councils, the Swedish Tobacco Foundation and INSERM and CNRS (France).

## REFERENCES

- AUBINEAU P, SERCOMBE R, EDVINSSON L & OWMAN CH 1977 Cholinergic influence on the cerebrovascular bed mediated through actions on the vascular smooth musculature and on the perivascular sympathetic nerves. In: Neurogenic control of the brain circulation (ed. Ch. Owman and L. Edvinsson), pp. 331-342. Pergamon Press, Oxford.
- EDVINSSON L, FALCK B & OWMAN CH 1977 Possibilities for a cholinergic action on smooth musculature and sympathetic axons in brain vessels mediated by muscarinic and nicotinic receptors. *J Pharmacol Exp Ther* 200: 117-126.
- EDVINSSON L, NIELSEN K C, OWMAN CH & SPORRONG B 1976 Cholinergic mechanisms in pial vessels. Histochemistry, electron microscopy and pharmacology. *Zellforsch* 134: 311-325 (1972).
- EDVINSSON L & OWMAN CH 1974 Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries *in vitro*. *Circulation Res* 35: 835-849.
- FARNEBO L-O 1971 On transmitter release evoked by field stimulation of monoamine neurons. P. A. Norstedt and Söner, Stockholm.
- NIELSEN K C, OWMAN CH & SPORRONG B 1971 Ultrastructure of the autonomic innervation apparatus in the main pial arteries of rats and cats. *Brain Res* 77: 3-32.
- OWMAN CH, EDVINSSON L & HARDEBO J E 1978 Pharmacological *in vitro* analysis of axon-mediated vasomotor functions in the intracranial and extracranial vascular beds. *Blood Vessels* 15: 128-147.
- SERCOMBE R, AUBINEAU P, EDVINSSON L, MAMO H, OWMAN CH, PINARD E & SEYLAZ, J 1975 Neurogenic influence on local cerebral blood flow. Effect of catecholamines or sympathetic nerve stimulation as correlated with the sympathetic innervation. *Neurology* 25: 954-963.
- SEYLAZ, J 1965 Développement d'une méthode de mesure instantanée localisée et semi-quantitative du débit sanguin *in vivo*. *Biophysik* 330-339.
- SEYLAZ, J, AUBINEAU P F, CORRÈZE, I L & MAMO H 1973 Techniques for continuous measurement of local cerebral blood flow.  $PaO_2$ ,  $PaCO_2$  and blood pressure in the nonanesthetized animal. *Pflügers Arch* 340: 175-180.
- SEYLAZ, J, PINARD E, CORRÈZE, I L, AUBINEAU P F & MAMO H 1974 Quantitative continuous measurement of blood gas tensions by mass spectrometry. *J Appl Physiol* 37: 937-941.

## Effects of tobacco smoking on the blood temperature during exercise

TOMAS BRUNDIN

Department of Clinical Physiology Huddinge Hospital S-141 86 Huddinge, Sweden

**ABSTRACT** By thermometry in mixed venous blood the thermal reactions to exercise programmes of different durations were studied in healthy volunteers before and after smoking. Pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ), arterio-venous oxygen difference (AVD) and heart rate (HR) were also measured. It was found that exercise-induced rise in blood temperature was considerably augmented after smoking. This effect was paralleled (and probably partly caused) by an increased heat production reflected by increased  $\dot{V}_{O_2}$ . During short-term exercise an accelerated rise in AVD might indicate that centripetal redistribution of the total blood flow could have contributed to the thermal response by reduced skin blood flow. During long-term exercise cardiac output and heart rate increased and the stroke volume decreased in response to smoking.

In studies on the blood temperature in healthy men during exercise remarkably high temperature values were sometimes noted in habitual smokers. Inspired by this observation, the present study was undertaken in order to find out whether tobacco smoking per se might interfere with the thermal adaptation to dynamic exercise.

By means of a technique developed for direct thermometry in mixed venous blood (Brundin 1975; Kilbom & Brundin 1976) the thermal response to exercise was measured in healthy individuals during standardized exercise programmes of different durations. The effect of tobacco smoking was studied by repeating the exercise programme after smoking. The results were compared with those from control groups who did not smoke between the exercise periods. The effects on pulmonary oxygen uptake, arterio-venous oxygen difference, cardiac output, stroke volume and heart rate were simultaneously studied.

### MATERIAL AND METHODS

**16 healthy volunteers.** —40 years of age, all habitual smokers, were studied. They came to the laboratory in the morning after light breakfast. They were not allowed to smoke for 12 h before the study. All the subjects were informed of the nature, purpose and possible risk in-

volved in the study before giving their voluntary consent to participate.

A teflon catheter (1.2 mm outer diameter) was inserted percutaneously under local anaesthesia into brachial artery. A thermistor-equipped catheter (Swan-Ganz 97-118 F, Edwards Laboratories) was inserted into an antecubital vein and passed to the pulmonary artery under fluoroscopic control.

In 8 of the subjects, mixed venous blood temperature (PA-temp), arterio-venous oxygen difference (AVD), and heart rate (HR) were measured at rest and during two identical programmes of short-term exercise performed with 30-40 min intermission. During the pause 5 subjects had to smoke 2 cigarettes before the second exercise period was started. 3 subjects served as controls and repeated the exercise programme without smoking.

Exercise was performed in the supine position on bicycle ergometer starting at work load of 20 W. The load was increased by 10 W each minute. The total exercise duration was 8 min and the final work load was 90 W. For some of the subjects the exercise was exhaustive or tiresome, the load being far below their maximum working capacity.

In 8 subjects, steady state exercise programme was used. Bicycle exercise was performed at constant work load of 90 W for 30 min. PA-temp, AVD, HR and pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ) were measured at rest, at 17-20 min and at 77-80 min after the onset of exercise. At 70-75 min, 5 of the subjects had to smoke 1 cigarette without interruption of the exercise before the final measurements. 3 of the subjects served as controls by performing the exercise programme without smoking.

The room temperature was kept around 20°C. The collected expiration air was analysed for oxygen and carbon

Table 1 Pulmonary oxygen uptake ( $\dot{V}_{O_2}$ ), arterio-venous oxygen difference (A\VD), cardiac output ( $\dot{Q}$ ), heart rate (HR) and stroke volume (SV) in 5 healthy volunteers at rest and during bicycle exercise at 50 W for 30 min

Smoking of 1 cigarette at 20–25 min of exercise. Values in brackets refer to control group = 3 exercising without smoking. Mean values  $\pm$  S.E.

	Exercise		
	Rest	17–20 min	27–30 min
$\dot{V}_{O_2}$ ml/min	263 $\pm$ 24 (280 $\pm$ 15)	790 $\pm$ 32 (828 $\pm$ 43)	$P<0.01$ 827 $\pm$ 25 (839 $\pm$ 39)
AVD ml O <sub>2</sub> /l blood	35 $\pm$ 1 (36 $\pm$ 1)	72 $\pm$ 3 (73 $\pm$ 3)	69 $\pm$ 3 (75 $\pm$ 4)
$\dot{Q}$ l/min	7.53 $\pm$ 0.63 (7.11 $\pm$ 0.18)	11.08 $\pm$ 0.83 (11.3 $\pm$ 0.70)	$P<0.05$ 11.13 $\pm$ 0.66 (11.26 $\pm$ 0.61)
HR, beats/min	65 $\pm$ 4 (70 $\pm$ 3)	98 $\pm$ 4 (109 $\pm$ 4)	$P<0.005$ 115 $\pm$ 6 (112 $\pm$ 3)
SV ml	117 $\pm$ 8 (102 $\pm$ 3)	113 $\pm$ 9 (104 $\pm$ 5)	$P<0.05$ 106 $\pm$ 7 (101 $\pm$ 3)

dioxide content according to Scholander (1947). Blood content of oxygen and carbon monoxide was measured by means of a semi-automatic oximeter (CO oximeter model 182, Instrumentation Laboratory Inc.). Cardiac output was calculated by the direct Fick method. Heart rate was followed by electrocardiographic recording. The thermistor signal from the pulmonary artery catheter was fed into an amplifier voltmeter-printer system and automatically recorded each second. The thermistors were calibrated after each study with a precision thermometer (Systemteknik AB) with an accuracy of  $\pm 0.01^\circ\text{C}$  (Brundin 1975).

Standard statistical method were used (Snedecor & Cochrane 1969) including the paired  $t$  test when applicable. Data in the text, tables and figures are reported as values  $\pm$  standard error of the means.

## RESULTS

### 1. Short term exercise

**Mixed venous blood temperature (PA-temp).** At rest the PA temp was within the normal range  $36.8\pm 0.2^\circ\text{C}$ . During exercise before smoking the blood temperature rise was similar in the control and experimental groups and similar to that previously found in other normal individuals (Brundin to be published). At 30 min after exercise the blood temperature values were not significantly different from those recorded at the initial baseline measurement. This was valid for both the experimental and the control group. After smoking the total exercise-induced temperature rise was almost 50% greater than that found before smoking (Fig. 1). There was no difference noted during the first minute of exercise, but after that, the temperature

increased considerably faster after smoking than before at all the work loads studied. In the control group of 3 subjects who did not smoke between the exercise periods almost identical temperature reactions were found at the two exercise periods (Fig. 1).

**Arterio-venous oxygen difference (A\VD).** At rest the AVD values  $39\pm 3$  ml/l were within the normal limits (cf. Ekelund & Holmgren 1967). During exercise the AVD increased in the same manner as that seen in other healthy individuals (Brundin to be published). After smoking the AVD at rest fell significantly as compared with the initial baseline measurement and was now  $29$  ml/l  $\pm 1$  indicating that cardiac output was increased by smoking. No such fall in AVD was observed in the control group.

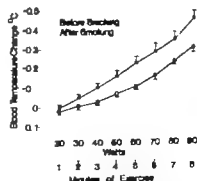


Fig. 1 Mean blood temperature change during short term exercise performed by 5 healthy volunteers before and after smoking of 2 cigarettes. Bars indicate S.E.

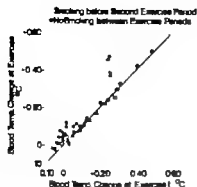


Fig. 2 Comparison of individual changes in blood temperature measured during repeated short term exercise periods. 5 subjects (●) smoked 2 cigarettes between the exercise periods 3 subjects (○) did not.

During exercise after smoking, however the AVD reached values similar to those observed during the first exercise period. Thus the exercise induced increase in AVD ( $\Delta$  AVD) was significantly greater after smoking. In the control group no such differences occurred.

The carboxy-hemoglobin in the arterial blood increased by around 2% after smoking and was not significantly changed during the second exercise period.

**Heart rate (HR)** At rest the mean HR was  $62 \pm 3$  beats per min. increased to  $116 \pm 10$  at 90 W and was 30 min after the first exercise period  $61 \pm 3$ . Smoking significantly increased the HR at rest to  $85 \pm 6$  and during the entire second exercise period the HR was significantly higher at each work load ending at  $130 \pm 8$  at 90 W. In the control group the HR reaction was almost identical during the two exercise periods.

## 2. Long-term exercise at steady state

**Pulmonary oxygen uptake ( $\dot{V}_{O_2}$ )** At rest before exercise  $\dot{V}_{O_2}$  was around 35% higher than the basal values calculated from body size. During exercise at 50 W  $\dot{V}_{O_2}$  was around 3 times higher than at rest. After smoking there was a further significant increase of around 5%. In the control group  $\dot{V}_{O_2}$  did not increase significantly during the last 10 min of exercise (Table 1).

**PA-temp** rose relatively steeply during the first 5 min of exercise and continued to rise more slowly up to 12 min. After that a steady state level was reached (Fig. 3). During smoking an additional temperature increase was recorded and a higher

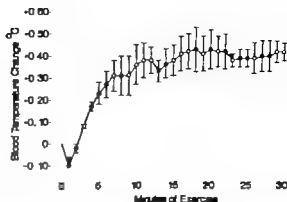


Fig. 3 Mean change in blood temperature during long term exercise (50 W) performed by 3 healthy volunteers. Bars indicate S.E.

steady state level was attained at 6 min after the onset of smoking (Fig. 4). This temperature increase was about 50% of the first temperature rise. In the control group the PA-temp was not significantly higher at 30 min than at 17 min of exercise.

**Arterio-venous oxygen difference (AVD)** showed a two-fold increase at 17 min of exercise and was not significantly changed by smoking (Table 1). In the control group the AVD change did not differ significantly from that of the experimental group.

**Cardiac output** calculated by the Fick equation, was significantly increased by around 10% after smoking. No similar difference was noted between 17 and 30 min of exercise in the control group (Table 1).

**Heart rate (HR)** reached a relative steady state level already within the first few minutes of exer-

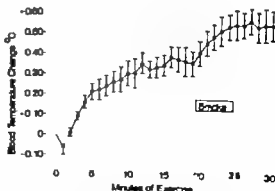


Fig. 4 Effect of smoking on the mean change in blood temperature during long term exercise (50 W) performed by 3 healthy volunteers. Bars indicate S.E.

cise. In the control group HR continued to increase very slowly towards the end of the 30 min exercise period. Smoking caused an abrupt increase in heart rate and a higher steady state value was attained during the continued exercise (Table I).

Stroke volume (SV) was not changed by exercise per se. However the markedly increased HR in response to smoking was more pronounced than the increase in cardiac output. Thereby the SV was significantly reduced in response to smoking. No such effect was observed in the control group.

The carboxy-hemoglobin content of the blood was significantly increased by around 1% after smoking.

## DISCUSSION

The relative hyperthermia observed in the blood during exercise after tobacco smoking seems not to have been described before. The effect appeared most markedly during short term exercise performed immediately after smoking. It could also be demonstrated as an increased steady state temperature level in direct response to smoking performed during a continuous period of long-term exercise.

During standardized bicycle exercise the blood temperature reaction, its rate of rise and its final steady state level is normally very well defined and easily reproducible (Brundin 1975, Kilbom & Brundin 1976). The results from the control groups turn that this was valid also for the actual experimental procedures. Thus the steeper blood

temperature rise and increased steady state temperatures found at exercise after smoking indicate that the balance between heat generation and heat elimination was interfered with by tobacco smoking. In case an increased heat generation constituted the main underlying mechanism for the relative hyperthermic reaction, an increased oxygen consumption would be expected to occur in parallel. The oxygen uptake could not be accurately measured during the short term exercise procedure. During exercise at steady state however smoking was followed by a small but statistically significant increase of the oxygen consumption. At rest nicotine has been reported to increase the metabolic rate by up to 10% (for ref. see Liebekk, Millder & Mjos 1975) but during exercise no such effect seems to have been demonstrated earlier. The increase in metabolic rate might constitute a direct metabolic response to the augmented sympatho-

adrenal activity which is known to occur in response to tobacco smoking (for ref. see Burn et al. 1959).

Although an increased heat generation could explain the relative hyperthermia during exercise after smoking, other mechanisms might well have contributed. Thermoregulation during exercise normally depends mainly on the capacity of heat elimination from the body which especially during short-term exercise will require an adequate increase of skin blood perfusion. There is no reliable method available for direct measurements of the total skin blood flow. However due to its low oxygen extraction from the blood, skin tissue gives a very oxygen-rich venous blood, the total amount of which will affect the arterio-venous oxygen difference (AVD) for the entire body. The rate of rise of the AVD-curve will thus be accelerated if the skin blood flow is reduced to any considerable extent. Such an acceleration was also found during short-term exercise after smoking. During steady state exercise however smoking caused no significant changes of the AVD. This might suggest that smoking was able to reduce the skin blood flow during its initial adjustment to dynamic exercise but was not able to change the level it had once attained during long-term exercise.

Cardiac output was found to increase significantly when smoking was performed during prolonged exercise at a steady state work load. As to the regional distribution of this increased blood flow the actual study does not allow for any direct suggestions. The increased adrenaline release which might follow smoking also during exercise could be expected to per se increase the cardiac output by regional flow increments in striated musculature, heart, splanchnic region and perhaps brain. However the concomitant increase of the sympathetic nerve activity and release of noradrenaline would interfere by vasoconstrictory action and the regional net effects would not be easily predictable.

The increase in heart rate observed was so marked that, in spite of the simultaneous increase in cardiac output, the stroke volume was significantly reduced when smoking was performed during the prolonged steady state exercise.

It is concluded that an increased thermal response to dynamic exercise can be added to the previously known pharmacological effects of tobacco smoking. During short term exercise the

relative hyperthermia response might have been reinforced by a centripetal redistribution of the total blood flow. During long-term exercise the temperature effect was paralleled by an increased heat generation reflected by a rise in total body oxygen consumption.

This study was supported by grants from the Swedish Tobacco Company.

## REFERENCES

- BRUNDIN T 1975 Temperature of mixed venous blood during exercise. *Scand J Clin Lab Invest* 35 339-343.
- BURN J H, LEACH E. H., RAND III J & THOMPSON J W 1959 Peripheral effects of nicotine and acetylcholine resembling those of sympathetic stimulation. *J Physiol (Lond)* 148 331-352.
- EKELUND L. G. & HOLMGREN A. 1967 Central haemodynamics during exercise. *Circulat Res* 20-21 Suppl 1 33-43.
- ILEBEKK A, MILLER N. E. & MÖS, O. B. 1975 Effects of nicotine and inhalation of cigarette smoke on total body oxygen consumption in dogs. *Scand J Clin Lab Invest* 35 67-72.
- KILBOM Å & BRUNDIN T 1976 Circulatory effects of isometric muscle contractions, performed separately and in combination with dynamic exercise. *Europ J Appl Physiol* 36 7-17.
- SCHOLANDER P. F. 1947 Analyses of accurate estimation of respiratory gases in one half cubic centimeter samples. *J Biol Chem* 167 235-250.
- SNEDECOR, G. W. & COCHRANE G. W. 1969 Statistical methods, 6th ed. Iowa State University Press Ames, Iowa 1969.



## Effect of nicotine on the formation of prostaglandins in the rabbit kidney

PAWEL ALSTER and ÅKE WENNMALM

Department of Clinical Physiology at Karolinska Institute  
Huddinge University Hospital S-141 86 Huddinge Sweden

**ABSTRACT** The effect of nicotine on the bioformation of prostaglandins (PG) in the rabbit kidney was investigated. Rabbit kidneys were homogenized and the low-speed supernatant was incubated with  $^{14}\text{C}$ -labelled arachidonic acid ( $^{14}\text{C}$  AA). PG precursor Nicotine was added to the incubations to produce final concentrations of  $5 \times 10^{-4}$  M to  $5 \times 10^{-3}$  M. Control incubations without nicotine were also performed.  $^{14}\text{C}$ -PG formed were separated and evaluated on thin layer radiochromatography. In control experiments  $^{14}\text{C}$ -labelled  $\text{PGD}_2$ ,  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  were formed, the relative amounts in between them being 16, 31 and 53 % respectively. Nicotine dose-dependently depressed the formation of all PG formed in the kidney. At  $5 \times 10^{-4}$  M the formation of  $^{14}\text{C}$  PG was depressed to 45-80 % of control and at a concentration of  $5 \times 10^{-3}$  M it was depressed to 40 % or less of control. It is concluded that nicotine elicits an overall depressive effect on the rabbit kidney formation of PG, probably by inhibiting cyclo-oxygenation of AA.

It has been demonstrated in our laboratory that nicotine interferes with the cardiac formation of prostaglandins (PG) (Wenmmalm & Justad 1975, Wenmmalm 1977). In these studies nicotine facilitated the efflux of PGE from the perfused rabbit heart. Later observations have indicated that nicotine selectively counteracts rabbit heart prostacyclin formation (Wenmmalm 1978a) and in parallel increases the amount of PGE formed in this organ (Wenmmalm 1978b). According to these experiments nicotine would rather than accelerating cardiac PG synthesis in general induce a change in the relative amounts of the different PG formed.

In the present study we have investigated the effect of nicotine in the rabbit kidney formation of  $\text{PGD}_2$ ,  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  and report that nicotine probably acts also at another level in the bioformation of PG, namely at the cyclo-oxygenation of arachidonic acid (AA).

### METHODS

Rabbit of mixed sexes and strains, weighing from 0.3-5 kg, were used. They were killed by blow on the head and exsanguinated via the carotid artery. The kidneys were rapidly removed, freed from fat tissue and homogenized in vol of 0.1 M potassium phosphate buffer pH 7.4. They

were subsequently minced and homogenized with teflon peston at 300 rpm and  $5^\circ\text{C}$  during 30 s. The supernatant thus obtained was used for the incubation experiments.

Supernatant corresponding to 1/10 rabbit kidney (vol 6 ml) was incubated with  $1.25 \mu\text{Ci}$  Na-arachidonate (prepared from  $^{14}\text{C}$  AA, New England Nuclear, sp.act. 40-60  $\text{mCi/mmol}$ ) for 5 min. Nicotine obtained from the chemical analysis laboratory at the Swedish Tobacco Company was dissolved in saline and kept at  $-18^\circ\text{C}$  in the dark. Immediately before the incubation the nicotine solution was added to the incubates to produce final concentrations of  $5 \times 10^{-4}$  to  $5 \times 10^{-3}$  M. Control incubations, in which no nicotine was added, were also performed. Incubation was interrupted by addition of 70 ml of water and adjustment of pH to 3.4.

Following decontamination of the incubation the acidified incubate was extracted twice with 25 ml of ethyl acetate. The organic phase was washed once with 4 ml of 0.5 M acetate buffer pH 6.5 and twice with 4 ml of water. It was subsequently dried on  $\text{Na}_2\text{SO}_4$ . After evaporation it was reconstituted in 0.1 ml of ethanol. Radiochromatographic separation was performed using 0.25 ml DC Kieselgel Fertigplatten F 254 (Merck) in solvent ethyl acetate:acetic acid 2:1, 4-trimethyl-pentane:water (90:20:10) / water phase discarded (ALX, Hamberg & Samuelsson 1966) against standards of  $\text{PGD}_2$ ,  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$ . Radioscans of the chromatograms from incubations using  $^{14}\text{C}$  AA substrate were developed with Berthold Durrnschmidt Scanner.

The relative amounts of the various  $^{14}\text{C}$ -labelled prostaglandins formed were calculated from the areas under the different radiopeak in relation to the total area of the



Table 1 Distribution of  $^{14}\text{C}$  PG formed from  $^{14}\text{C}$  AA in supernatant of homogenized rabbit kidney

The value for a single PG is expressed in % of the total amount of PG formed

	$\text{PGD}_2$	$\text{PGE}_2$	$\text{PGF}_{25}$
Mean	16	31	53
S.E.	2	2	10
	4	7	7

identified peaks in the chromatograms. Values in text, table and figures are presented as mean  $\pm$  S.E.

## RESULTS

Incubation of the supernatant of the centrifuged kidney homogenate with  $^{14}\text{C}$  Na-arachidonate resulted in formation of various labelled compounds which were readily distinguishable in the radio-scans. Major peaks were found in parallel to the AA,  $\text{PGD}_2$ ,  $\text{PGE}_2$  and  $\text{PGF}_{25}$  standards. No other radio-peaks were obtained regularly. The relative quantitative distribution of the  $^{14}\text{C}$  PG-peaks obtained in control experiments is shown in Table 1.  $^{14}\text{C}$   $\text{PGF}_{25}$  was the major compound formed followed by  $^{14}\text{C}$   $\text{PGE}_2$  and  $^{14}\text{C}$   $\text{PGD}_2$ .

Addition of increasing doses of nicotine to the incubates induced a progressive decrease in the

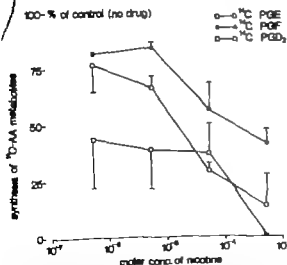


Fig. 1 Supernatant of kidney homogenate incubated with  $^{14}\text{C}$  Na-arachidonate and different concentration of nicotine. The symbols indicate mean  $\pm$  S.E. of the various  $^{14}\text{C}$ -PG in % of the amount formed in control incubations without drug.

Distribution of  $^{14}\text{C}$  AA metabolites

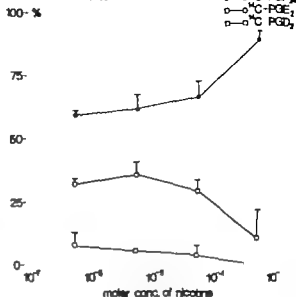


Fig. 2 Distribution of the  $^{14}\text{C}$  PG formed in supernatants of kidney homogenate incubated with  $^{14}\text{C}$ -Na-arachidonate and various concentration of nicotine. The symbols indicate mean  $\pm$  S.E. of the various  $^{14}\text{C}$  PG in % of the total amount of  $^{14}\text{C}$ -PG formed at that concentration of nicotine

amount of  $^{14}\text{C}$  PG formed. Thus the formation of  $^{14}\text{C}$   $\text{PGF}_{25}$  which at a nicotine concentration of  $5 \times 10^{-7}$  M was about 80% of control (without drug) decreased to about 40% of control at a nicotine concentration of  $5 \times 10^{-4}$  M. The formation of  $^{14}\text{C}$   $\text{PGE}_2$  was likewise depressed from about 75% of control at  $5 \times 10^{-7}$  M to about 15% at  $5 \times 10^{-4}$  M. The formation of  $^{14}\text{C}$   $\text{PGD}_2$  was most sensitive to the inhibitory action of nicotine. At  $5 \times 10^{-4}$  M the amount of  $^{14}\text{C}$   $\text{PGD}_2$  formed was only about 45% of control and at  $5 \times 10^{-3}$  M no formation of  $^{14}\text{C}$   $\text{PGD}_2$  was observed (Fig. 1). The relative distribution of the  $^{14}\text{C}$  PG formed varied with increasing concentrations of nicotine.  $^{14}\text{C}$   $\text{PGF}_{25}$  increased from 57% in controls to 89% at a nicotine concentration of  $5 \times 10^{-4}$  M and  $^{14}\text{C}$   $\text{PGE}_2$  decreased from 33% in controls to 11% at  $5 \times 10^{-4}$  M. The formation of  $^{14}\text{C}$   $\text{PGD}_2$  was rapidly depressed being only 8% of the total  $^{14}\text{C}$  PG formed at  $5 \times 10^{-4}$  M.

## DISCUSSION

The present experiments demonstrate that increasing concentrations of nicotine elicit a dose-related inhibitory effect on the formation of  $^{14}\text{C}$  PG formed

from  $^{14}\text{C}$  AA in rabbit kidney homogenates. This effect of nicotine is not completely uniform. Thus the formation of  $\text{PGD}_2$  is apparently very sensitive to the inhibitory action of nicotine: the amounts of  $^{14}\text{C}$   $\text{PGD}_2$  formed being depressed by more than 50% at a nicotine concentration of  $5 \times 10^{-4}$  M and completely inhibited at  $5 \times 10^{-3}$  M. The synthesis of  $^{14}\text{C}$   $\text{PGE}_2$  is also strongly depressed by nicotine at a concentration of  $5 \times 10^{-4}$  M being decreased to about 15% of control. The formation of  $^{14}\text{C}$   $\text{PGF}_{2\alpha}$  is the most insensitive to the inhibitory action of nicotine: at  $5 \times 10^{-4}$  M the synthesis was still about 40% of control. These data seem to indicate a difference in sensitivity to the inhibitory effect of nicotine of the pathways from PG endoperoxides to  $\text{PGD}_2$ ,  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  respectively. Such a difference appears even more evident if the distribution of  $^{14}\text{C}$  PG formed in the presence of different concentrations of nicotine is calculated as in Fig. 2. In that diagram the relative increase in the formation of  $^{14}\text{C}$   $\text{PGF}_{2\alpha}$  at higher concentrations of nicotine is evidently visible.

Although these differences in sensitivity to nicotine apparently exist in the kidney formation of various PG, the most significant finding of this investigation is that nicotine depresses the total amount of PG formed from AA. This strongly suggests that nicotine acts at an enzymatic level that is common to the formation of all PGs i.e. on the cyclo-oxygenation of AA to PG endoperoxide. The observed differences in sensitivity to nicotine of the formations of PG D, E, and F probably merely reflect that the dominating metabolic pathway (leading to formation of  $\text{PGF}_{2\alpha}$ ) on restriction of the amounts of PG endoperoxides formed retains an increasing fraction of the amount of substrate (PG endoperoxide).

In conclusion nicotine affects the formation of PG in the supernatant of rabbit kidney homogenates considerably. The various metabolic pathways for PG synthesis in this preparation display marked differences in sensitivity to the inhibitory effect of nicotine which makes the pharmacological significance of the data uncertain. In inhaling cigarette smokers the nicotine concentration in plasma has been reported to be about  $5 \times 10^{-6}$  M (Armitage et al. 1974). In the current experiments that concentration produced a 50% inhibition of the formation of  $\text{PGD}_2$ . The possibility that cigarette smoking affects PG formation in the human body therefore seems likely and deserves increased attention.

This study was supported by the Swedish Tobacco Company and by the Swedish Medical Research Council (project 04X-4341). Prostaglandin standards were kindly put at our disposal by Dr J. Pike, Upjohn Company.

## REFERENCES

- ARMITAGE, A. K., DOLLERY, C. T., GEORGE, C. F., HOUSEMAN, T. H., LEWIS, P. J. & TURNER, D. M. 1974 Absorption and metabolism of nicotine by man during cigarette smoking. *Brit. J. Clin. Pharmacol.* **1**, 180P-181P.
- HAMBERG, M. & SAMUELSSON, B. 1966. Prostaglandins in human seminal plasma. *J. Biol. Chem.* **241**, 257-263.
- WENNMALM, Å. 1977. Nicotine stimulates prostaglandin formation in the rabbit heart. *Br. J. Pharmacol.* **59**, 95-100.
- WENNMALM, Å. 1978a. Nicotine inhibits the release of 6-keto-prostaglandin  $\text{F}_{1\alpha}$  from the isolated perfused rabbit heart. *Acta Physiol. Scand.* **103**, 107-109.
- WENNMALM, Å. 1978b. Effects of nicotine on cardiac prostaglandin and platelet thromboxane synthesis. *Br. J. Pharmacol.* **64**, 559-563.
- WENNMALM, Å. & JUNSTAD, M. 1976. Nicotine mediated release of prostaglandin E from the rabbit heart. *Acta Physiol. Scand.* **96**, 281-282.



## Psychological effects of tobacco smoking

DAISY SCHALLING and DICK WALLER

Department of Psychiatry Karolinska Hospital and Department of Psychology University of Stockholm, Stockholm, Sweden

The main purpose of the psychological studies of tobacco smoking is to elucidate the mechanisms underlying the peculiarly persistent habit of inhaling into the mouth and lungs the smoke from burning tobacco. What are the psychological and pharmacological factors interacting in the starting and maintaining of such a habit?

The complicated question of smoking motivation has been approached in many different ways. The simplest way to find out why people smoke would seem to be to ask them. However it is difficult to obtain valid information on motives by direct questions. To overcome this problem, indirect approaches have been tried. How do people rank a set of possible motives? Which of several possible changes in mood and alertness are experienced most often? In what situations or moods do people tend to smoke or feel like smoking? Another indirect approach to smoking motivation is to study the personality traits of heavy smokers. Who are the people who get addicted to smoking?

Such approaches have been used in some studies on smoking motivation performed by students in the Department of Psychology, University of Stockholm (Hultberger 1974, Waller 1975). Some of their findings will be briefly reviewed here, as a background to the main topic for the present paper—a review of some studies of objective effects of tobacco smoking on mental performance (attention or vigilance) and on psychophysiological and pain measures, interpreted in terms of arousal regulation or reward effects.

### *Personality traits of heavy smokers*

It has been consistently found that amount of smoking tends to be associated with the personality traits of extraversion, impulsiveness, and monotony avoidance (sensation-seeking), and psychopathy, estimated by personality inventory scales (Schalling 1977). Impulsivity refers to behavioural tendencies like acting on the spur of the moment, lack of anticipation or planning, and the seeking of variety and thrill. These personality traits are among the

few for which biological correlates have been consistently found. Among these are indices of a low level of reticular-cortical arousal (Schalling 1978), e.g. a low frequency of spontaneous fluctuations in skin conductance, and some EEG characteristics. It is possible that the smoking of heavy smokers may constitute a link in the self-regulation of arousal, one easily available method of increasing the capacity to sustain attention. This interpretation is rendered more likely by the known effects of nicotine on release of acetylcholine (Holmstedt & Lundgren 1967) which appears to be related to increased cortical arousal (Warburton 1975).

### *Smoking motive and subjective effects of smoking*

Waller (1975) studied smoking motivation in several groups of male moderate smokers. The subjects tended to rank motives related to pleasurable and calming effects highest. The two most frequently reported effects of smoking were an increase in ability to relax and to concentrate. However the individual variations were substantial.

The more neurotic or anxiety-prone subjects reported that they were smoking predominantly in order to achieve calmness. However increased irritability and tension were the main effects reported by these subjects. These seemingly paradoxical reports may be interpreted as reflecting irrational behavior in neurotic persons. However for persons with habitually high arousal, a further increase may lead to a supraoptimal arousal state (Eysenck 1967).

### *Smoking in high arousal and low arousal limit*

Desire for smoking obviously varies with the situation. Applying a list of different situations, Hultberger (1974) found higher ratings for smoking desire in low arousal situations (e.g. relaxing at home) than in high arousal situations (e.g. waiting for a difficult examination) which is in line with English findings, using the same list. A classification of smokers on the basis of this list into high-

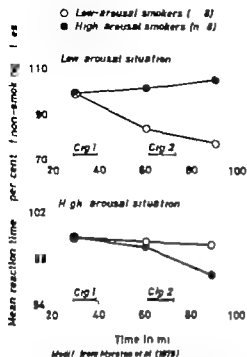


Fig. 1. Mean reaction time in smoking sessions in per cent of non-smoking values for eight low-arousal smokers (open circles) and eight high-arousal smokers (filled circles) (from Myrsten et al. 1975).

arousal smokers and low arousal smokers yielded interesting sex and personality differences. Female subjects were predominantly high-arousal smokers, male subjects low-arousal smokers. The low-arousal smokers had conspicuously low scores in a Psychic Anxiety scale measuring a tendency to worry and anticipate (Schalling 1977). Performance differences were reported (Fig. 1) between high-arousal and low arousal smokers selected in a similar way. In line with the predictions on the basis of this classification (Myrsten, Andersson, Frankenhaeuser & Mårdh 1975).

However, Waller (1975) using a modified situation list in which the arousal value for each item had been determined empirically by ratings, found higher smoking desire in high-arousal than in low-arousal situations in a group of male moderate smokers.

#### Laboratory studies of acute effects of tobacco smoking

In a recent study from our research group the effect of tobacco smoking on critical flicker fusion (CFF; the fusion frequency of flickering light) was measured by a new method: a computerized forced-choice interactive technique (Waller & Levander

1979). This measure reflects the number of impulses that the retinal-cortical system can process in a unit of time and it is regarded as an indicator of CNS efficiency or cortical arousal. Subjects were 28 male moderate smokers who participated in a Smoking (S) and a Non Smoking (NS) condition, each of one hour duration. CFF was measured during 15 two min trials in each condition. In the S condition the level of CFF was studied intermittently during 15 min before smoking, during 15 min when CFF trials were interspersed with periods in which subjects smoked three puffs on a cigarette of their preferred brand and during 30 min after smoking.

There was a marked improvement in CFF performance with a maximum 8 min after the first puff (Fig. 2). Performance was significantly higher in the S condition compared to the NS condition from five min after the first puff to 20 min after the last puff. Analysis of variance was made for CFF performance averaged over trials for a presmoking period (1) a smoking period (2) and a postsmoking period (3). There was a significant condition  $\times$  period interaction ( $p < 0.1$ ). In Period 1 the performance was almost identical in the S and NS condition. In Period 2 performance was markedly improved in the S condition followed by a slight decline in Period 3. In the NS condition there was a slight impairment in performance over periods. Order of conditions did not affect the outcome. It is of interest that the duration of the effects corresponds to the known duration of increased CNS activation after smoking as indicated by EEG. The smoking induced improvement in ability to discriminate visual stimuli may be taken as an indicator of an increased level of cortical arousal.

In order to explore the large individual differences in effects of smoking found in this and other studies, two extreme groups were formed consisting of the subjects most and least improved in the S condition. The improved group had significantly higher scores in an extraversion scale ( $p < 0.05$ ).

In another laboratory study from our group skin and stance was recorded during seven successive rest periods surrounding short pain tolerance measurements. In the group of subjects who were allowed to smoke, spontaneous fluctuations showed a marked increase but of very short duration (Fig. 3) most pronounced during the first minute of smoking. The interpretation of this preliminary finding is not clear. The postganglionic mediation of skin conductance responses is cholinergic

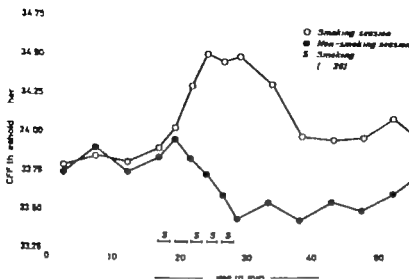


Fig 2 Mean CFF performance during 11 successive measurements in a smoking condition and non-smoking condition ( $n=28$ )

while the central and preganglionic mediation is believed to be both adrenergic and cholinergic. Skin conductance spontaneous fluctuations are related to reticular stimulation in animal research (Bloch 1965) and have been associated with level of cortical arousal in human subjects (Schalling 1978). Thus the marked effects of smoking in our study may be related to an acute acetylcholine mediated phasic increase in reticular-cortical arousal.

Finally some preliminary results will be given from a study of the effects of tobacco smoking on experimental pain response. Increased pain toler-

ance after smoking has been reported (Nesbitt 1973). In a recent study from our group experimental pain responses were studied in 4 subjects (8 male and 16 female) during smoking and non-smoking conditions, using electrocutaneous stimulation (Mueser Waller Levander & Schalling 1978). Pain thresholds and tolerance levels were obtained with a method of limits (ML). Further a sensory decision analysis was made on the effects of smoking on ability to discriminate between electrical stimuli of different intensity (P (A) a non-parametric measure corresponding to  $d'$  and related to sensory

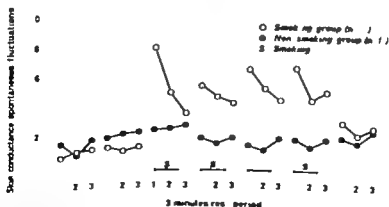


Fig 3 Mean number of skin conductance spontaneous fluctuations during the first, second and third minute in seven successive three minute periods for smoking group ( $n=11$ ) and non-smoking group ( $n=11$ )

components of pain) and the effects on response criteria (B: a measure related to non-sensory motivational factors). There were significant sex differences in the smoking-induced change in ability to discriminate strong stimuli: males being somewhat improved, females impaired.

A more detailed analysis was made on the 8 male subjects and an age-matched group of 8 female subjects. Analysis of variance on ML pain tolerance levels during the smoking and non-smoking conditions yielded a significant interaction with sex ( $P < 0.01$ ) and with scores in the Psychic Anxiety scale ( $P < 0.01$ ). Smoking increased pain tolerance levels in male subjects and independently of sex in subjects low in Psychic Anxiety.

The smoking induced increase in discrimination ability in the male subjects may be related to nicotine effects on cholinergic reticular transmission systems (Warburton 1975). However the apparently paradoxical findings of an increased tolerance of pain stimulation in male subjects implies another additional mechanism of action. It has been suggested that release of endorphines may be facilitated by smoking in some subjects. The spectacular results of acupuncture in heavy smokers during smoking cessation therapy which have been reported also in some controlled studies (Lacroix & Besançon 1977) may imply endorphine release as one of the mechanisms behind tobacco addiction. However it is obviously too early to draw conclusions and much more research is needed in these areas.

#### Concluding comments

The mediation of the pleasure and reward-related and "analgesic" effects of smoking reported above may be pharmacologically different from the mediation of the effects on reticular-cortical arousal suggested by the CFF and skin conductance findings.

There are wide individual differences in smoking effects and an important task for the biopsychological study of smoking is to further explore these individual differences and their psycho-

physiological and biochemical correlates. In this way it might be possible to elucidate the role of smoking in the self-regulation of arousal and mood.

#### REFERENCES

- BLOCH V 1965 Le contrôle central de l'activité électrodermale. Etude neurophysiologique d'un indice réticulaire. *Journal de Physiologie (Paris)* Suppl. 13: 1-132.
- EYSENCK H J 1967 Biological basis of personality. Thomas Springfield, IL.
- HOLMSTEDT B & LUNDGREN B 1967 Arecoline, nicotine and related compounds. Tremorogenic activity and effect upon brain acetylcholine. *Ann NY Acad Sci* 142: 126-142.
- HULTBERGER M 1974 Rökvanor i relation till personlighetsvariabler och situationvariabler (Smoking habits as related to personality and situational variables). Unpublished thesis, University of Stockholm.
- LACROIX J & BESANÇON F 1977 Le sévrage du tabac. Efficacité de l'acupuncture dans un essai comparatif. *Ann Med Intern* 128: 403-408.
- MUESER, K., WALLER D, LEVANDER, S. & SCHALLING D 1978. Smoking and pain—a method of limits and sensory decision theory analysis. Manuscript.
- MYRSTEN A, L. ANDERSSON R, FRANKENHAEUSER M & MÄRDH A 1975 Immediate effects of cigarette smoking as related to different smoking habits. *Perceptual and Motor Skills* 40: 515-523.
- NESBITT P D 1973 Smoking, physiological arousal and emotional response. *Journal of Personality and Social Psychology* 25: 137-144.
- SCHALLING D 1977 The trait-situation interaction and the physiological correlates of behavior. In *Personality at the crossroads, current issues in international psychology* (ed. D. Magnusson & N. S. Endler). Erlbaum (Wiley), Hillsdale, NJ.
- SCHALLING D 1978 Psychopathy-related personality variables and the psychophysiology of socialization. In *Psychopathic behaviour: Approaches to research* (ed. R. D. Hare & D. Schalling). Wiley, Chichester.
- WALLER, D 1975 Studier av rök-motivation. (Studies in smoking motivation.) Unpublished thesis, University of Stockholm.
- WALLER, D & LEVANDER S 1979 Smoking and vigilance. The effects of tobacco smoking on CFF as related to personality and smoking habits. Preliminary manuscript.
- WARBURTON D M 1973 *Brain, behaviour and drugs*. Wiley, London.







ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 480

**Muscle fatigue in man**  
with special reference to lactate accumulation  
during short term intense exercise

By  
PER TESCH

STOCKHOLM 1980



ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 480

From the Department of Clinical Physiology Karolinska sjukhuset and  
Department of Physiology III Karolinska institutet Stockholm, Sweden

# Muscle fatigue in man

with special reference to lactate accumulation  
during short term intense exercise

By  
PER TESCH

STOCKHOLM 1980



## CONTENTS

INTRODUCTION	5
SUBJECTS	6
METHODS	8
Statistics	8
Force measurement	8
Maximal oxygen uptake	9
Cycling exercise	9
Downhill skiing	9
EMG recordings	10
Muscle biopsy sampling	10
Histochimistry	10
Biochemistry	12
Blood analysis	13
RESULTS AND COMMENTS	13
Muscle fibre type distribution in vastus lateralis of man	13
Muscle strength and its relation to muscle fibre type distribution	14
Muscle power and fatigue and the relation to fibre type distribution	15
Lactate accumulation in different muscle fibre types	17
Lactate metabolism and muscle fatigue	18
LDH and its significance for lactate formation	20
Myoelectric activity during fatiguing conditions	21
Lactate formation during downhill skiing	23
GENERAL DISCUSSION	24
Metabolic profiles of muscle fibre types in man	24
Muscle fibre recruitment	24
Muscle exercise and metabolism	26
Force generation and motor unit activity	26
Lactate accumulation and muscle fatigue	28
Central versus peripheral fatigue	29
SUMMARY	30
ACKNOWLEDGEMENTS	31
REFERENCES	32

The present thesis is based on the following papers which will be referred to by their Roman numerals

- I Teuch P B Sjödin and J Karlsson Relationship between lactate accumulation LDH activity LDH isoenzyme and fibre type distribution in human skeletal muscle Acta physiol scand 1978 103 40-46
- II Teuch P B Sjödin A Thorstensson and J Karlsson Muscle fatigue and its relation to lactate accumulation and LDH activity in man Acta physiol scand. 1978 103 413-420
- III Nilsson J P Teuch and A. Thorstensson Fatigue and EMG of repeated fast voluntary contractions in man Acta physiol scand 1977 101 194-198
- IV Rodi P V and P Teuch. EMG frequency spectrum muscle structure and fatigue during dynamic contractions in man. Eur J Appl Physiol 1979 42 41-50
- V Teuch P Fatigue pattern in subtypes of human skeletal muscle fibres Int. J Sports Med. Accepted for publication
- VI Teuch P Local lactate and exhaustion. Acta physiol scand. 1978 104 373-374
- VII Teuch P L Larsson A Eriksson and J Karlsson Muscle glycogen depletion and lactate concentration during downhill skiing Med Sci Sports 1978 10 85-90

In addition some unpublished results are included

## INTRODUCTION

The feeling of discomfort and pain due to heavy muscular exercise is an experience with which most individuals are familiar. Housework, occupational work, leisure as well as sport activities will sometimes lead to these states which can be referred to as fatigue experience. A conclusive definition of the fatigue phenomenon does not exist (cf. Bartley & Chute 1947, Bartley 1965, Simonson 1971) although the question of its origin has been the subject of controversy among researchers in various scientific fields during more than half a century. Moiso (1890) who constructed and used a finger ergograph was one of the first to report changes in mechanical performance during repeated muscle contractions. Since then muscular fatigue as indicated by a failure to generate maximum voluntary force or a prescribed force level, has been extensively studied in order to establish its possible sites of origin (e.g. Lee 1906, Cobb & Forbes 1923, Reid 1928, Brown & Burns 1949, Merton 1954, Haas & Storm-Mathisen 1955, Bohrerer & Bourguignon 1959, Lippold et al. 1960, Stephens & Taylor 1972, Bigland-Ritchie et al. 1978).

Interest has also been focused on the behaviour of different motor unit types including their extrafusal muscle fibres in terms of fatigability. Recent review articles concerning specific characteristics of different motor units in animal (Close 1972, Burke & Edgerton 1975) as well as man (Saltin et al. 1977) are available. As already reviewed by Needham (1926) the classical "red" muscle possesses a greater resistance to fatigue as compared to the "white" muscle. By means of a mechanical definition of motor units, Nachbolder (1931) demonstrated that tonic muscles are more fatigue resistant as compared to phasic muscles. Later this pattern has been confirmed in a number of animal studies (e.g. Eberstein & Sander 1961, 1963, Kugelberg & Edström 1968, Burke et al. 1971). In fact the property in this respect has been included in the fibre type nomenclature (cf. Burke & Edgerton 1975) in order to distinguish between fast twitch (FT) muscle fibres with different fatigability levels.

Recently it has been possible also in man to relate sustained (Nilbech & Johansen 1973, Saltin et al. 1975) and repeated (Ochs et al. 1977) isometric contraction fatigue directly or indirectly to the histochemical profile of the activated muscle. Using a device to study dynamic contractions (Parrinello 1968) based on the force-velocity principle as introduced by Hill (1938), Thorstensson (1976) was able to demonstrate a positive relationship between the individual percentage of fast twitch (type II) muscle fibres in the vastus lateralis muscle and the decrease in force output during repeated knee extensions.

Attempts have been made to explain the basic features of fatigue by relating metabolic and mechanical changes which occur during exercise (Hill & Kupalov 1930). Since the work of Fletcher & Hopkins (1907) many authors have suggested lactate accumulation as directly or indirectly causing an impaired muscular function (e.g. Berg 1936, Amundsen et al. 1948, Karlsson 1971, Karlsson et al. 1975). Some electrophysiologists who have studied changes in action potential conduction



and the frequency patterns of motor units agree on this suggestion (e.g. Lindström et al. 1970; Mortimer et al. 1970). Other experimental observations however speak in favour of a block at the neuromuscular junction (Stephens & Taylor 1972) as the site of muscle fatigue.

It is generally believed that at the onset of heavy muscular activities adenosine triphosphate (ATP) and creatine phosphate (CP) stored in the muscle exclusively will cover the energy demand. Theoretically however these immediate energy sources can be depleted in man within 10 seconds (Margaria et al. 1964). Such short periods of maximal exercise concomitantly result in the formation of lactate (Pernow & Mehren 1962; Hultman et al. 1967; Bergström et al. 1971; Saltin et al. 1971). Simultaneously signs of muscle fatigue or impairment can be recognized as indicated by a decrease in mechanical output within this time limit. In order to elucidate the fatigue phenomenon, the present study was designed by combining established histochemical, biochemical and electrophysiological methods when examining high intensity exercise of short duration. More specifically experiments were carried out:

- a. To study physically active males with muscles differing in muscle fibre type distribution with regard to performance capacity during short term dynamic exercise of high intensity.
- b. To analyse lactate concentration in pools of different muscle fibre types following short term intense exercise.
- c. To study electromyographic (EMG) patterns with emphasis on changes in frequency spectrum of motor units during exercise as described above.

#### SUBJECTS

A total of 54 subjects was studied. Age, height and weight averaged 24 (range 19-42) yrs, 180 (range 170-194) cm, and 72 (range 60-89) kg. They were all physically active and most were physical education students or subjects with a similar background. Among these were 14 individuals participating in more regular physical training programs and competing on the national elite level in cycling, long-distance running, orienteering and downhill skiing. In certain cases subjects were involved in more than one experiment. Major physical characteristics of the subjects are summarized in Table 1. Prior to giving their oral consent to participate in the study, the subjects were informed of the purpose and the risks associated with the testing procedures. The investigation was approved by the Ethical Committee at Karolinska Institutet, Stockholm.

Table 1 Characteristics of subject groups Mean ( $\pm$ SE) values or ranges are given Maximal oxygen uptake was determined using exercise on a cycle ergometer

Study	n	Age yrs	Height cm	Weight kg	$\dot{V}O_2$ max $\text{ml} \times \text{kg}^{-1} \times \text{min}^{-1}$	Category
I	10	24.1	179-1	71 $3.2 \pm 1$	59 $1.1 \pm 9$	Physical education students
II	9	23-1	179-1	69 $9.2 \pm 8$	60 $5.1 \pm 4$	—
III	12	22-1	180-1	70 $3.1 \pm 6$	—	Physical education students Endurance trained
IV	11	25-2	179 $^{+1}$	72 $0.1 \pm 9$	59 $1.1 \pm 8$	Physical education students
V	23	24.1	180-1	72 $2.1 \pm 7$	—	Physical education students Endurance trained
VI	7	22-1	180-1	69 $3.2 \pm 0$	65 $5.1 \pm 3$	Endurance trained
VII	8	24-1	178-1	69 $9.1 \pm 8$	—	Physical education students
	3	20-22	174-182	72-76	—	Downhill skiers
	2	28-42	170-177	66-75	—	Skid school instructors
	8	24-1	181.1	74 $2.1 \pm 5$	—	Physical education students

and the frequency patterns of motor units agree on this suggestion (e.g. Lindström et al. 1970; Mortimer et al. 1970). Other experimental observations however speak in favour of a block at the neuromuscular junction (Stephens & Taylor 1972) as the site of muscle fatigue.

It is generally believed that at the onset of heavy muscular activities adenosine triphosphate (ATP) and creatine phosphate (CP) stored in the muscle exclusively will cover the energy demand. Theoretically however these immediate energy sources can be depleted in man within 10 seconds (Margaria et al. 1964). Such short periods of maximal exercise concomitantly result in the formation of lactate (Pernow & Wahren 1962; Bultman et al. 1967; Bergström et al. 1971; Saltin et al. 1971). Simultaneously signs of muscle fatigue or impairment can be recognized as indicated by a decrease in mechanical output within this time limit. In order to elucidate the fatigue phenomenon the present study was designed by combining established histochemical, biochemical and electrophysiological methods when examining high intensity exercise of short duration. More specifically experiments were carried out:

- a To study physically active males with muscles differing in muscle fibre type distribution with regard to performance capacity during short term dynamic exercise of high intensity
- b To analyse lactate concentration in pools of different muscle fibre types following short term intense exercise
- c To study electromyographic (EMG) patterns with emphasis on changes in frequency spectrum of motor units during exercise as described above

## SUBJECTS

A total of 54 subjects was studied. Age, height and weight averaged 24 (range 19-42) yrs, 180 (range 170-194) cm, and 72 (range 60-89) kg. They were all physically active and most were physical education students or subjects with a similar background. Among these were 14 individuals participating in more regular physical training programs and competing on the national elite level in cycling, long-distance running, orienteering and downhill skiing. In certain cases subjects were involved in more than one experiment. Major physical characteristics of the subjects are summarized in Table 1. Prior to giving their oral consent to participate in the study the subjects were informed of the purpose and the risks associated with the testing procedures. The investigation was approved by the Ethical Committee at Karolinska Institutet, Stockholm.

Muscle fatigue was expressed as the absolute as well as the relative torque decline from the initial peak torque and defined as "fatigue index". A high test-retest reliability for the fatigue test has earlier been demonstrated (Thorstensson 1976). A coefficient of variation of 3.2% was obtained based on day-to-day measurements. In order to evaluate whether subjects in the present study did exert MPT in the fatigue test, initial peak values from a fatigue test were compared with MPT values as well as initial peak values obtained in a repeated fatigue test some days later. The c.v. corresponded to 3.7 and 3.9% (n = 22) respectively. In addition, 10 subjects were re-tested after 18 months. During the time elapsed, physical activity was maintained at an almost constant level comparable to the pre-test level. The reproducibility of the fatigue test, expressed as c.v., was 6.9% while the corresponding value found for MPT was 5.3%.

#### Maximal oxygen uptake (I, II, IV, VI)

Maximal oxygen uptake was determined using leg exercise on a Monark cycle ergometer by stepwise increasing the work load according to the levelling off criterion (Åstrand & Saltin 1961). Expired air was collected in Douglas bags and the volume measured in a spirometer. For  $O_2$  and  $CO_2$  analyses a mass spectrometer (Centronic 200 MGA, Centronic Models, Croydon, England) was used. Duplicate determinations revealed a s.e.v. of 1.2% (n = 20).

#### Cycling exercise (VI)

To make comparisons possible with previous muscle fatigue studies, short term maximal cycle exercise was applied. This took place without any warm up period. An exhaustive cycling test was performed on a Monark cycle ergometer with a work load corresponding to 170 (range 118-121) % of  $\dot{V}O_{2\max}$ . Inability to maintain a prescribed pedalling frequency (i.e. 70 rev  $\times$  min<sup>-1</sup>) was taken as criterion for exhaustion. The day-to-day variation in exercise tests of this kind (n = 10) was 3.1% (c.v.).

#### Downhill skiing (VII)

To study the effect of short term heavy muscle exercise in sport events, subjects performed submaximal and maximal skiing of approximately 1 min duration on a slope adjusted for competition.

The glycogen depletion pattern (Gollnick et al. 1972a) was studied during two consecutive days of skiing in order to evaluate muscle fibre recruitment pattern. The exercise consisted of ski-school activity (physical-education students) and competitive slalom and giant slalom training (elite skiers). Training sessions for both groups lasted approximately 5 h per day.

### EMG recordings (III-IV)

To evaluate changes in electromyographic activity during the muscle fatigue experiments EMG was registered from the vastus lateralis muscle. Surface electrodes (Hellige Stockholm) of 10 mm in diameter were placed over the belly of the muscle as close as possible to the site of the muscle biopsy insertion. Muscle biopsies (see later) were taken at least one week prior to EMG experiments. The interelectrode distance was 10 mm in the direction of the patella-crista iliaca.

Amplified EMG signals were either rectified and filtered by a single lag filter (120 ms time constant) and displayed on a UV Honeywell 2012 recorder along with the corresponding torque curve or amplified and immediately stored on magnetic tape (Philips Analog 7) with a recording speed of  $380 \text{ mm} \times \text{s}^{-1}$  for further analysis. Peak EMG was defined as the highest point on the rectified and filtered EMG curve while integrated EMG (IEMG) was taken as the area under the rectified and filtered EMG curve as measured by planimetry. Analysis was limited to the mid-range of knee extension.

Using a laboratory computer (HP 2116 C) and a data processing system (Romi & Lehtis 1973; Viitasalo & Romi 1975) IEMG and power spectral density function (PSDF) were obtained according to Bendat & Piersol (1971). In order to demonstrate changes in PSDF mean power frequency (MPF) was computed and the relative proportions were calculated for selected bandwidths (24-48 Hz, 56-88 Hz, 96-128 Hz, and 136-400 Hz). For further description see Viitasalo & Romi (1977).

### Muscle biopsy sampling (I-VII)

Muscle tissue specimens (approximately 30-50 mg) were taken from vastus lateralis using the percutaneous needle biopsy technique (Bergström 1962). This part of the quadriceps muscle has been found to be heavily activated when performing knee extensions during isokinetic conditions (Herrifield & Dozal 1978), maximal cycling exercise (Bigland-Ritchie & Woods 1974; Henriksen & Bunde-Petersen 1974) and downhill skiing (Eriksson et al. 1976).

Biopsies for histochemistry were mounted in embedding medium (Cryofom<sup>TM</sup> Denson Needham Hs Mass) and frozen in isopentane cooled with liquid nitrogen. For biochemistry muscle biopsies were frozen directly in liquid nitrogen. Samples were stored at  $-80^{\circ}\text{C}$  until analysed. Specimens for glycogen determinations, histochemical as well as biochemical were obtained within 5 min before and after exercise. Muscle biopsies for lactate assays were obtained within 3-5 s after exercise with the subjects in a sitting position while muscle biopsies after skiing were taken within 10-15 s in a supine position.

### Histochemistry (I-VII)

Serial transverse sections (10  $\mu\text{m}$ ) were cut in a microtome at  $-25^{\circ}\text{C}$ . Stainings were undertaken for ATPase activity (Gomori 1941; Padykula & Hansen 1955) after preincubation at pH 10.3, 4.6 and 4.3 respectively (Engel 1962; Brooks & Kaiser 1970) as well as for NADH-tetrazolium reductase (Novikoff et al. 1961). For classification fibres were

identified as fast twitch (FT) and slow twitch (ST) fibres (Engel 1962) and for further subgroup classification of FT fibres (Brooke & Kaiser 1970) into FTs and FIB according to the terminology proposed by Saltin et al. (1977). The percentage of each fibre type was calculated from sections containing at least 200 fibres but mostly comprising 400-500 fibres. Muscle fibre area was determined on NADH-tetrazolium reductase stained fibres using a cutting and weighing procedure (Thorsteinsson 1976). At least 10 fibres of each fibre type subjectively rated as representative for the entire transverse sections were selected for analysis. The relative muscle area occupied by FT muscle fibres was calculated according to the formula:  $(100 \times \text{FT/ST area} \times \% \text{ FT}) \times (\text{FT/ST area} \times \% \text{ FT} + \% \text{ ST})^{-1}$ . Error of methods (c.v.) estimated from analysis of duplicate biopsies ranged 6-11% for area and distribution variables (Table 2).

Table 2 Methodological errors (c.v.) of histochemical and biochemical variables. Samples used for lactate assay ranged 10-35 in concentration (mmoles  $\times \text{kg}^{-1}$  wet muscle). Analyses were conducted on pools of fibres ranging 10-30  $\mu\text{g}$  in dry weight. Mean values were calculated from 2-3 pools of fibres and therefore each value represents a dry weight corresponding to approximately 40-80  $\mu\text{g}$ .

<u>HISTOCHEMISTRY</u>	<u>c.v. %</u>	<u>n</u> <sup>2)</sup>
Muscle fibre type distribution <sup>1)</sup>	6.2	28
Muscle fibre area <sup>1)</sup>	10.5	30
Muscle fibre area ratio <sup>1)</sup>	7.5	26
<u>LACTATE</u>		
<u>ANALYSES UNDERTAKEN SIMULTANEOUSLY</u>		
Pool to pool unidentified fibres	6.8	35
— FT fibres	8.7	35
— ST fibres	7.2	35
— divided fibres	3.6	12
Mean to mean, unidentified fibres	4.4	32
— FT fibres	4.4	12
— ST fibres	4.4	12
<u>ANALYSES UNDERTAKEN ON TWO SUCCESSIVE DAYS</u>		
Pool to pool divided fibres	6.7	14
Mean to mean, —	2.2	6
<u>ANALYSES UNDERTAKEN ON DIFFERENT OCCASIONS</u>		
Mean to mean	5.6	12

1) Duplicate biopsies

2) Duplicate determinations

To demonstrate selective glycogen depletion pattern cross-sections (16  $\mu$ m) were PAS (periodic acid-Schiff) stained for glycogen (Pearse 1961). Individual fibres were subjectively rated as filled, moderately emptied and exhausted using a system similar to that proposed by Gollnick et al. (1972a).

## Biochemistry (I, II, VI, VII)

Metabolites were determined in mixed muscle samples as well as in separated, dissected out fibres. After weighing at  $-25^{\circ}\text{C}$  on a Cahn electrobalance, muscle biopsies (approximately 10–50 mg) were analysed for their glycogen and lactate contents by means of fluorometer techniques according to Lowry & Passonneau (1972) as modified by Karlsson (1971) using a Farrand ratio fluorometer model 24 (Farrand Optical Co., New York).

Analyses on separate muscle fibres were performed in the following way: after lyophilizing muscle samples (approximately 2–12 mg) were placed under a microscope and 100–200 fragments of muscle fibres were dissected out from each sample at a constant room temperature and humidity ( $20^{\circ}\text{C}$  and 30% respectively). Thereafter a part of each fibre was cut off and stained for fibre type identification (Esaén et al. 1975a). For lactate analysis 5–13 FT and ST fibre fragments respectively were pooled and weighed on a Cahn electrobalance or Quartz fibre ultramicro balance (Rodder "E" Microtech Services, Berkeley). Weight of the samples corresponded to 10–30  $\mu\text{g}$ . Fibres were then put into test tubes before the reagent solution was added. The lactate concentration was determined according to Karlsson (1971). Each value was calculated as a mean value for the lactate concentration in 2–7 pools. Comparing values obtained from conventional analysis on whole wet (Karlsson 1971) or freeze-dried (Esaén 1978) samples with the present method, no systematic differences were noted in terms of lactate concentration, although wet sample values tended to be lower than confirming Karlsson (1971). Other methodological errors for lactate analysis estimated from duplicate determinations are listed in Table 2. All values were converted into wet weight values assuming a water content of 77% for muscle biopsy samples (Karlsson 1971). This value for muscle biopsy water content was confirmed and no differences were obtained irrespective of whether biopsies ( $n = 25$ ) were taken at rest or after short term (60 s) heavy exercise. However, small intra- and interindividual variations in water content were present, corresponding to 75–78%.

In order to express lactate concentration for the entire muscle investigated, the following formula was applied: Lactate concentration in FT fibres  $\times$  % FT area + lactate concentration in ST fibres  $\times$  % ST area. Lactate dehydrogenase (LDH) activity and muscle specific LDH (M-LDH) activity for the entire muscle were calculated in a manner corresponding to lactate.

For muscle tissue enzyme studies, additional dissected pools of FT and ST fibres, each consisting of approximately 100 fibre fragments, were homogenized in 100  $\mu\text{l}$  of 0.5 M KCl. Total lactate dehydrogenase (LDH) (E.C. 1.1.1.27) activity in the forward reaction (pyruvate  $\rightarrow$  lactate,  $\text{LDH}_{\text{tot}}$ ) was determined fluorometrically.

(Lowry & Passonneau 1972) in the homogenate diluted 1:4 with 0.1 M Tris-HCl buffer pH 7.5. Discelectrophoresis for separating the LDH isoenzymes was performed according to Dietz & Lubrano (1967) on 25  $\mu$ l of the homogenate. The relative contribution of muscle specific LDH isoenzymes (% M-LDH) was determined using a densitometric scanning technique on the separated and stained LDH isoenzyme bands in the gels (Sjodin 1976). The activity corresponding to the muscle specific LDH (M-LDH) was then calculated (% M-LDH  $\times$  LDH<sub>tot</sub>). The error of method (c.v.) estimated from duplicate biopsies was 3.1% (n = 10) (Sjodin, personal communication).

### Blood analysis

Blood samples were obtained from the finger tip and analysed for lactate concentration according to the Barker and Summerson method as modified by Ström (1949).

## RESULTS AND COMMENTS

### Muscle fibre type distribution in vastus lateralis of men (I-VII)

Muscle fibre type distribution in the vastus lateralis muscle in 64 healthy young men averaged 49 (range 10-79) % FT fibres. The relative area occupied by FT fibres averaged 52 (range 10-77) %. When subjects involved in competitive physical training were excluded, the values corresponded to 53 (range 29-79) % FT and 57 (range 28-77) % FT area respectively. Individual FT/ST muscle fibre area averaged in the whole material 1.20 (range 0.70-1.85) and 1.24 (range 0.88-1.85) when trained subjects were excluded (Table 3).

Table 3 Mean (range) values for muscle fibre type distribution and areas in the sample studied including (n = 64) and excluding 14 individuals involved in competitive physical sports

	Fibre type distribution % FT	FT fibre area 100 $\mu$ m <sup>2</sup>	ST fibre area 100 $\mu$ m <sup>2</sup>	FT area %	FT/ST area
n 64	49 (10-79)	64.5 (35.9-101.7)	58.4 (29.4-86.9)	52 (10-77)	1.20 (0.70-1.85)
n 50	53 (29-79)	66.8 (35.9-101.7)	55.8 (29.4-86.9)	57 (28-77)	1.24 (0.88-1.85)



Subgroup classification of FT muscle fibres was undertaken on samples from 23 subjects. The mean distribution of PTA and PTb fibres was 30 (range 13-49) % and 19 (range 0-37) % respectively.

Comments: The subjects were recruited among physical education students or individuals with approximately an equivalent physical activity level. Fourteen athletes involved in competitive physical training were also included.

The results concerning fibre type distribution and fibre area in *m. vastus lateralis* of men revealed a pattern in agreement with previous studies (Edström & Ekblom 1972; Gollnick et al. 1972b). Although there is a wide range in the distribution, muscles of physically active though not competitive men seem to be composed of a smaller proportion of FT fibres as compared to muscles of individuals in the sedentary state as also reported by e.g. Gollnick et al. (1972b) and Larsson (1978). Furthermore, it was noted that individuals successful in endurance events displayed a more homogeneous fibre type distribution characterized by a high percentage of ST fibres and a low occurrence of PTb fibres, thus confirming other authors (Jansson 1975; Rygaard-Jensen 1976; Jansson & Kaijser 1977; Jansson et al. 1978).

Muscle strength and its relation to muscle fibre type distribution (I-V)

Peak torque, attained at a relatively high speed of movement ( $180^{\circ} \times s^{-1}$ ), either expressed per kg body weight or in absolute terms, was positively correlated to the percentage of FT fibres as well as to the relative proportion of FT fibres ( $r = 0.48-0.65$ ,  $p < 0.001$ ,  $n = 50$ ). In addition, peak torque was related to the absolute FT fibre area ( $r = 0.30-0.33$ ,  $p < 0.05$ ).

In a subgroup ( $n = 18$ ) peak torque at a higher angular velocity ( $300^{\circ} \times s^{-1}$ ) was studied. This speed is approximately 50% of the maximum angular velocity that could be obtained during knee extension (Thorstensson 1976). Peak torque averaged 131 Nm or  $1.8 \text{ Nm} \times \text{kg}^{-1} \text{ b.w.}$  as compared to 151 Nm or  $2.1 \text{ Nm} \times \text{kg}^{-1} \text{ b.w.}$  at  $180^{\circ} \times s^{-1}$ . The observed relation between the proportion of FT fibres and peak torque developed was not further strengthened by increasing angular velocity. Approximately the same correlation coefficients were found when relating peak torque to percentage of FT fibres as well as to the relative area occupied by FT fibres. No relationship could be demonstrated between peak torque and the relative occurrence of one or the other type of FT fibre subgroups.

Comments: Peak torque developed at movements of relatively high angular velocities ( $180-300^{\circ} \times s^{-1}$ ) was found to be related to the percentage of FT fibres, thus confirming Thorstensson & Karlsson (1976). Since peak torque also was related to the absolute FT fibre area, but not to mean fibre area, there are reasons to believe that the quality of the activated muscle, i.e. the proportion of FT fibres, is of primary importance for force development at higher angular velocities. This suggestion is in accordance with recent observations by Coyle et al. (1979). Consequently, the quantity, i.e. the muscle mass as such, is suggested to be of secondary importance in terms of

force development at high angular velocities. Even though it has been possible to correlate mean fibre area with the cross-section of the muscle by means of dector tomography (Nygaard et al. 1978a, Høggmark et al. 1978) such an interpretation must however be taken with caution.

#### Muscle power and fatigue and the relation to fibre type distribution (II-VI)

Decrease in peak torque was found to occur within 10 maximal muscle contractions ( $p < 0.001$ ,  $n = 50$ ). Muscle fatigue either expressed as the relative or absolute decline in peak torque after 50 contractions was positively correlated to the muscle fibre type distribution expressed as % FT ( $r = 0.43-0.61$ ,  $p < 0.01-0.001$ ) or % FT area ( $r = 0.40-0.58$ ,  $p < 0.01-0.001$ ) ( $n = 50$ ). The relationship observed between fibre type distribution and relative decline in peak torque confirms the findings by Thorstensson (1976). In a subsample muscle fatigue was found to be related also to the occurrence of Ftb muscle fibres (Fig. 1). Approximately the same correlation coefficients were present when relating the decline in peak torque either expressing the fibre type population as above ( $r = 0.53-0.57$ ,  $p < 0.01$ ) or to the percentage of Ftb fibres ( $r = 0.55-0.57$ ,  $p < 0.01$ ).

Changes in peak torque were as expected accompanied by corresponding changes in power and work. Hence decline in power and work was correlated to the percentage of FT fibres ( $r = 0.64-0.75$ ,  $p < 0.05-0.01$ ,  $n = 12$ ). The amount of work produced during the first 15 contractions (20 s) was approximately 20% greater ( $p < 0.05$ ) in a group of subjects ( $n = 5$ ) with muscles made up by a high proportion of FT fibres (mean  $\pm$  SE  $62 \pm 4\%$ ) as compared to individuals ( $n = 5$ ) with a low proportion of FT fibres (37-3%). With additional contractions this difference became smaller and smaller. After 100 contractions the correlated amount of work was approximately equal comparing the two groups (Fig. 2).

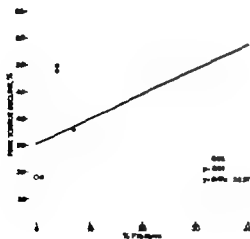


Fig. 1. The relationship between the percentage of Ftb muscle fibres in the vastus lat. ralis muscle and the decline in peak torque with 50 maximal knee extension at an angular velocity of  $180^\circ \times s^{-1}$  ( $\text{rad} \times s^{-1}$ ) ( $n = 23$ ). Individual with muscle fibre type distribution less than 35% FT fibres are denoted with open (O) dots.

LDH and its significance for lactate formation (I II VI unpublished results)

Correlation coefficients between LDH<sub>tot</sub> as well as M-LDH activity and individual muscle fibre type distribution (% FT) were 0.96 ( $p < 0.001$ ) and 0.98 ( $p < 0.001$ ) respectively. In addition, the relative contribution of M-LDH in FT and ST fibres respectively was related to the percentage of FT fibres in the muscles investigated ( $r = 0.74$ ,  $p < 0.01$  and  $r = 0.89$ ,  $p < 0.001$ ) i.e. the proportion of FT fibres in the muscle reflected the quality of the ST fibre in respect to its LDH activity.

To study further the significance of LDH enzyme activity on lactate formation capacity, 10 subjects performed 25 repeated muscle contractions according to Thorsen et al. (1976). Mean values were 18.5 (range 3.5–31.1)  $\mu\text{moles} \times \text{kg}^{-1}$  wet weight (lactate), 0.90 (range 0.38–1.65) LDH<sub>tot</sub> and 0.60 (range 0.18–1.54) (M-LDH)  $\mu\text{moles} \times \text{kg}^{-1} \times \text{min}^{-1} \times 10^4$  wet weight. The individual differences in muscle lactate accumulation after exercise were found to be related to individual variations in LDH<sub>tot</sub> ( $r = 0.66$ ,  $p < 0.05$ ) and M-LDH ( $r = 0.70$ ,  $p < 0.05$ ) activity. This relationship was also present in 7 endurance trained individuals. At exhaustion following a short term cycling exercise, the individual variation in lactate concentration (mean and range were 20.8 and 13.9–30.5  $\mu\text{moles} \times \text{kg}^{-1}$  wet weight) was found to be positively correlated to the percentage M-LDH ( $r = 0.73$ ,  $p < 0.05$ ) (Table 4).

**Comments:** The data confirm earlier findings by Karlsson et al. (1974) and Sjödin (1976) that muscle fibre type distribution will determine the metabolic quality of the muscle in terms of its potentials for lactate formation. In addition to this, an interaction appears to be present between the two muscle fibre types. Thus, in ST fibres of muscles rich in FT fibres, the LDH activity as well as LDH isozyme pattern approached the properties of FT fibres more than in a muscle rich in ST fibres. Whether or not this is an effect of adaptive mechanisms due to the environment remains speculative.

Table 4. The interrelationship between some physiological, histochemical, biochemical and functional parameters obtained in 5 individuals performing short term high intensity exercise.

Subject	$\dot{V}O_{2\text{max}}$ $\text{ml} \times \text{kg}^{-1} \times \text{min}^{-1}$	PT %	M-LDH %	Lactate concentration $\mu\text{moles} \times \text{kg}^{-1}$ wet muscle			Performance time s
				FT	ST	whole muscle	
I	70.0	29	39	14.4	13.6	13.8	99
II	65.9	29	45	26.2	15.5	18.8	101
III	66.6	40	39	26.7	21.4	23.8	119
IV	60.6	42	56	26.0	23.5	24.8	124
V	61.0	62	74	31.8	26.5	30.5	126

Taken together the data from the isokinetic one leg and cycling experiments seem to indicate that the activity and quality of the LDH-enzyme are critical for lactate formation and performance capacity. Previously it has been demonstrated that physiological concentrations of lactate *in vitro* will decrease LDH activity (Karlsson et al. 1974). Furthermore it has been shown that phosphofructokinase (PFK) activity considered to be the rate limiting enzyme for glycolysis is inhibited at low pH (Demforth 1965). A decrease in pH has been demonstrated concomitant to lactate formation in voluntary contracting human muscle (Sahlin 1978).

#### Myoelectric activity during fatiguing conditions (III-IV)

Although a pronounced decrease in peak torque was demonstrated after 50 and 100 contractions corresponding to 50-75% peak EMG and IEMG decreased only slightly ( $n = 1$ ) in the whole material studied ( $n = 23$ ). In a subsample of individuals ( $n = 6$ ) with muscles with a high proportion of FT fibres IEMG decreased significantly ( $p < 0.01$ ). As a consequence EMG activity for the whole material either expressed as peak EMG per unit peak torque or IEMG per unit work increased progressively to level off around the 75th contraction. The change (increase) in these ratios with 50 contractions was positively correlated to individual muscle fibre type distribution expressed as per cent FT fibres ( $r = 0.84$   $p < 0.001$  and  $r = 0.78$   $p < 0.01$ ). Consequently the increase in the IEMG/torque ratio was positively related to the relative decrease in peak torque ( $r = 0.88$   $p < 0.001$ ) following 100 contractions.

Different qualitative EMG changes were observed during repeated muscle contractions. Thus a 25% decrease ( $p < 0.001$ ) in power spectral density function (PSDF) of the EMG expressed as mean power frequency (MPF) was present in a group of individuals ( $n = 6$ ) with muscles rich in FT fibres (mean  $\pm$  SE relative FT fibre area  $67 \pm 34$ ). In a group of subjects ( $n = 5$ ) with muscles not as rich in FT fibres ( $43 \pm 14$ ) a smaller nonsignificant decrease of MPF was present. Intergroup deviations were noted already after 25-30 contractions ( $p < 0.01$ ). When these two groups were combined, individual relative change in MPF after 25 contractions was positively related to peak torque decline ( $r = 0.74$   $p < 0.01$ ) as well as to muscle fibre type distribution expressed as per cent FT fibres ( $r = 0.60$   $p < 0.05$ ).

Comparing the frequency spectrum changes between the two groups certain differences were observed. Increase in the relative content of the lower bandwidth (24-48 Hz) as well as decrease in the higher bandwidth (136-400 Hz) were significantly greater ( $p < 0.01$  and  $p < 0.05$  respectively) in subjects characterized by muscles rich in FT fibres.

In addition to qualitative changes in EMG activity events linked to the neuromotoric control were found to be altered with a concomitant reduction in muscle force. Thus individual differences related to muscle fibre type distribution were noted when studying the time lag between the first action potential of the EMG and the corresponding torque recording at a pre-set angular velocity ( $180^\circ \times s^{-1}$ ) ( $n = 12$ ). Values demonstrated initially and after 50 and 100 contractions were (mean  $\pm$  SE)

$95 \pm 3$ ,  $115 \pm 5$  and  $121 \pm 5$  ms respectively. The time lag was initially negatively related to the percentage of FT fibres ( $r = -0.68$ ,  $p < 0.05$ ) and the percentage time lag increase after 100 contractions was positively related to % FT ( $r = 0.73$ ,  $p < 0.01$ ).

**Comments:** The fatigue associated shift in EMG frequency spectrum towards low frequency components is in accordance with Kogi & Hakarada (1962). A significant drop in EMG activity as well as a more pronounced shift in the frequency spectrum of EMG with fatigue was noted in muscles with a high percentage of FT fibres as compared to muscles with a high percentage of ST fibres. Although information regarding lactate concentration was not available from these experiments, it can be assumed that after 25 contractions higher values were present in those subjects who demonstrated the most pronounced shifts in PSDF. This reasoning seems to hold true since data obtained on individuals also taking part in experiments where lactate concentration was determined, revealed an inter-relationship between force decline, lactate accumulation (and LDH activity) and PSDF shift (Fig. 5). Whether changes in time lag between onset of EMG and torque development at a pre-set angular velocity depend on biochemical processes taking place as a result of repeated muscle contractions cannot be established here. Nevertheless, the present data clearly indicate that the muscle rich in FT fibres initially has the shortest time lag but suffers more during repeated contractions as compared to the muscle rich in ST fibres.



Fig. 5. Change (expressed as % of initial values) of two individual in peak torque, integrated EMG (IEMG) and mean power frequency (MFF) during repetition of 100 maximal knee extensions. Muscles biopsies of lactate concentration ( $\text{mmol} \cdot \text{s}^{-1} \cdot \text{kg}^{-1}$  wet muscle) determinations were obtained in separate experiment. The two subjects differed in respect to muscle fiber type distribution; subject GC (●): 54% FT fibres and subject GL (○): 34% FT fibres.

### Lactate formation during downhill skiing (VII)

In order to study the effects of acute physical activity in sports demanding great muscular power downhill skiing was used as an experimental model. Low muscle glycogen values in the vastus lateralis muscle were noted after a day of skiing and in two samples studied levels below 15 mmol/kg glucose units  $\times$  kg<sup>-1</sup> wet weight were found. Skilled and unskilled skiers demonstrated different selective glycogen depletion patterns. In both groups a great loss of glycogen from the FT fibres was demonstrated. However, in the skilled skiers a pronounced depletion of ST fibres was observed as well. Based on these findings it was suggested that downhill skiing to a high degree recruited the FT fibre population irrespective of training status. This was supported by the fact that it was not possible to relate muscle lactate accumulation to level of proficiency. However, lactate concentration in the exercised muscle was positively correlated to the relative distribution of FT muscle fibres after maximal skiing (Fig. 3). No such relationship was demonstrated following submaximal skiing conditions resulting in significantly lower lactate values. Furthermore, the FT/ST lactate ratio reached higher values during maximal skiing in individuals rich in FT fibres as compared to their opposites ( $r = 0.84$ ,  $p < 0.05$ ).

Comments: Low muscle glycogen levels were demonstrated after skiing and such values are only reported after prolonged intense running, cycling or cross-country skiing. Thus it can be concluded that downhill skiing, competitive as well as recreational, is performed with an enhanced utilization of glycogen, especially in FT fibres, and that the site of the quadriceps muscle chosen is representative to study muscle metabolism during downhill skiing.

Downhill skiing is evidently a physical activity which is dependent on recruitment of FT fibres irrespective of training status and is therefore in some respects comparable to the different laboratory tests applied in the present study. Accordingly it suggests that the FT fibres were more active in formation of lactate. It can be suggested that downhill skiing, especially in less skilled skiers, will be characterized by a high degree of isometric muscle exercise. This assumption is supported by the fact that a high FT/ST lactate ratio was observed in subjects rich in FT fibres, which is in accordance with findings obtained during standardized isometric exercise at different tension levels (Toech & Karlsson 1977).

The present data indicated that skilled skiers recruited relatively more of their ST fibre population than unskilled. Rysgaard et al. (1978b) observed pronounced glycogen depletion of Pfb fibres in one inexperienced skier while none or minor loss of glycogen from these types of fibres was observed in some advanced skiers. This provides further evidence of a contrasting recruitment pattern in skilled and unskilled individuals.

## GENERAL DISCUSSION

## Metabolic profiles of muscle fibre types in man

Studies on separated human skeletal muscle fibre types disclose a consistent pattern regarding their metabolic profiles. Thus in terms of metabolic potentials the ST fibre is suited for use in prolonged activities with reliance upon oxidative metabolism, indicated by higher activity of enzymes involved in the citric acid cycle e.g. succinate dehydrogenase (Essén et al. 1975a, Henriksson & Reitman 1976) as well as a LDH isoenzyme pattern favouring lactate oxidation (Sjöödin 1976). Furthermore observations on habitually active men have indicated a greater blood flow in muscles rich in ST fibres as compared to muscles rich in FT fibres (Juhlin-Varnfalt et al. 1979). Thus a relationship between leg vascular resistance and muscle fibre type distribution was demonstrated which fits well with the greater capillary density of muscles rich in ST fibres (Andersen 1975, Andersen & Henriksson 1977). Moreover a relationship between the mitochondrial content and number of capillaries surrounding each fibre has been demonstrated (Brodehl et al. 1977). The FT fibre on the other hand in addition to superiority in terms of contractile characteristics (Thorstensson et al. 1977) display higher activity of enzymes thought to be rate limiting for glycolysis i.e. phosphorylase (Piehl & Karlsson 1977), phosphofructokinase (Essén et al. 1975a, Henriksson & Reitman 1976) and lactate dehydrogenase (Sjöödin 1976, Thorstensson et al. 1977). Even in terms of stored metabolites differences are present between the two main fibre types. Slightly higher CP concentrations were noted in FT fibres while no differences in ATP concentration of fibre types were noted in the resting muscle (Essén 1978). Triglyceride content is significantly higher in ST fibres (Essén et al. 1975a) whereas glycogen content seem to be approximately equal in different fibre types (Essén et al. 1975a, Piehl & Karlsson 1977).

In addition the subgroups of FT fibres are known to differ in terms of enzymatic and vascular profile. In most respects the FTa fibre seems to represent an intermediate type in comparison to the ST and FTb fibre (cf. Saltin et al. 1977).

Muscle fibre type distribution as determined by staining for myofibrillar ATPase in the muscle reflects the acto-myosin ATPase activity which in turn is assumed to be correlated with intrinsic speed of muscle shortening (Börjesson 1967, Börjesson & Closs 1971) in the investigated muscle. Considering this the present results clearly demonstrate that individual fibre type distribution to a certain extent will be important for anaerobic performance capacity as indicated by repeated muscle contractions of high intensity.

## Muscle fibre recruitment

Established methods are available for evaluation of the muscle fibre recruitment pattern in vivo. The degree of glycogen depletion in different fibre types by histochemical means is one method to evaluate whether a certain type of fibre has been metabolically active during exercise (Rugelberg & Ekström 1968).

Gollnick and co-workers (Gollnick et al 1973a b 1974a b) found with this approach that ST fibres seem to be recruited at muscle contractions calling for less than 20% of maximal voluntary isometric contractions. At tensions exceeding 20% FT muscle fibres are recruited in addition. In dynamic (cycling) exercises with an energy need below maximal oxygen uptake ( $\dot{V}O_2 \text{ max}$ ) ST muscle fibres are the first to loose glycogen. At supra-maximal load corresponding to 120% of  $\dot{V}O_2 \text{ max}$  muscle glycogen depletion pattern indicates an initial loss from FT fibres although there are reasons to believe that a major portion of the ST fibre population is recruited simultaneously. The rate of pedalling ( $30-120 \text{ rev} \times \text{min}^{-1}$ ) does not influence the glycogen depletion pattern. Moreover at intensities close to or above  $\dot{V}O_2 \text{ max}$  both FT fibre types are recruited (Andersen & Bjørgaard 1976). However it is not possible to establish with this approach whether certain muscle fibre types are continuously involved during an entire pedal thrust or engaged only in short bursts. Using the same experimental isokinetic exercise protocol as in the present study contraction velocity was found to influence muscle fibre recruitment pattern suggesting a greater involvement of FT fibres at faster velocities (Leaves et al 1979).

Intramuscular substrates other than glycogen may have been utilized as indicated by Essén (1978) analysing single muscle fibres for their metabolic contents. Furthermore a higher energy consumption per unit isometric tension developed has been demonstrated for FT as compared to ST fibres of hamster (Goldspink et al 1970) which may obscure the recruitment pattern as indicated by glycogen staining intensities. In line with this is the finding that the rate of heat production in human skeletal muscles is 6 times higher in FT as compared to ST fibres (Boilestad & Ekstrand 1978). The rate of glycogen breakdown is also found to partly be dependent on individual muscle fibre type distribution (Ahlén-Dannfelt et al. 1977) and consequently care must be taken when evaluating for fibre recruitment pattern using the glycogen depletion method.

In order to evaluate the orderly recruitment pattern of single motor units electromyography using fine needle electrodes (Henneman 1974) and bipolar surface electrodes (Gydikov & Rosarov 1974) has been studied. Information on muscle fibre recruitment pattern in man has been obtained in isometric (cf Gydikov & Rosarov 1974 Grimby & Henneman 1977) but also in isotonic (Grimby & Henneman 1977) leg exercises. The results confirm the size principle theory based on experiments on decerebrated cat experiments (Henneman et al 1965). In addition, an initial selective recruitment of FT motor units was demonstrated during ballistic movements irrespective of muscle tension level (cf Grimby & Henneman 1977). These results indicate that an exclusive pool of FT motor units always will fire at onset of a muscle contraction when muscle tension is rapidly increased and/or forceful. FT fibres were also observed to be activated upon sudden and vigorous contractions in partially denervated human muscles (Marcolita & Engel 1972).



## Muscle exercise and metabolism

The present findings indicate that it is possible to objectively detect a decrease in the individual's ability to produce maximum muscle force within 10 muscle contractions. For such a short period of intense exercise no experimental data are available showing complete depletion of the immediate energy stores. A certain reduction of muscle ATP and CP depots are however present after 10-15 s of heavy exercise (Bultman et al. 1967, Bergström et al. 1971, Saltin et al. 1971).

The question may be raised whether or not a complete lack of ATP in the myofibrillar regions is possible. Based on frog and rat experiments impairment of contractile function due to inadequate availability of myofibrillar ATP has been suggested by e.g. Pitts & Holloszy (1976, 1977). Murphy (1966) stimulating frog sartorius muscles demonstrated a close relationship between tension output and the ATP content of a metabolically inhibited muscle. However he concluded that it is unlikely that any ATP dependent limitations will be present in the mechanical response of muscles with intact ATP restitution mechanisms.

Spande & Schottelius (1970) stimulated isolated mouse muscle to fatigue and observed a linear relationship between CP concentration in the muscle and tension developed in an isometric tetanus. They proposed that CP depletion can be the underlying cause of fatigue. Whether the contractile machinery was directly affected or not could not be established from their results.

Information concerning the glycogen depletion pattern during brief exercise in the order of 10-25 contractions are not available but most probably muscle contractions irrespective of contraction velocity and tension output must be repeated for longer periods of time in order to exhaust certain fibres (Andersen & Sjøgaard 1976, Secher & Nygaard Jensen 1976, Essén 1978).

It is open to speculation whether exhaustion following supramaximal cycling exercise is partly attributed to substrate depletion of highly recruited FF fibres. By repeating exhaustive supramaximal one minute bouts approximately 50% of the FF fibre population was found to be exhausted of their glycogen content already after 5 bouts (Thomson et al. 1979). Since this fibre type to a high extent seems to contribute to the force applied at each pedal thrust (see above) selective substrate depletion cannot be excluded as being a limiting factor in intense cycle exercises in the order of 1-2 min duration.

## Force generation and motor unit activity

In order to study the origin of fatigue changes in EMG activity and muscle force have been followed during various type of activity resulting in impairment of muscle function. During sustained constant submaximal isometric contraction increased EMG activity occurs (Merton 1954, Edwards & Lippold 1956, Lippold et al. 1960, Ruzda et al. 1970, Viitasalo & Komi 1977). This has been assumed to be due to recruitment of additional motor units and/or increased firing frequency to compensate for failure in contractility of already recruited fibres (Lippold et al. 1960). In experiments

where the fatigue mechanism was studied in sustained maximal isometric contraction in man a fall in EMG activity concomitant to a loss in muscle force was observed (Stephens & Taylor 1972). The unchanged electrical activity to tension (E/T) ratio was taken as indicative of fatigue at the neuromuscular junction. Ochs et al. (1977) demonstrated also a drop in electrical activity when studying EMG and force relationship during rapid repetitive maximal isometric plantar flexion. In comparison with Stephens & Taylor (1972) muscle tension was however found to decrease at a higher rate as compared to the EMG activity which implies an increased E/T ratio. Hence muscle fatigue was indicated to be located peripheral to the neuromuscular junction.

Dissimilarities regarding changes in E/T ratio during development of muscle fatigue may be influenced by factors such as type of contractions investigated (e.g. submaximal vs. maximal isometric contraction, isometric vs. dynamic contraction, slow vs. fast dynamic contraction). It has also been suggested that migration of activity may occur within a muscle as well as from one muscle to another (Lippold 1955, Komi & Viitasalo 1976).

Studying human single motor units a decrease in firing frequency (Marras et al. 1971) has also been noted concomitantly with a fall in muscle tension (Gydlkov & Kozarov 1974) indicating that EMG might fall without any neuromuscular block. Contradicting findings regarding E/T ratio and conclusions in the literature might be due to differences in type of motor units dominating in the investigated muscle. By comparing the EMG activity of the human gastrocnemii and soleus known to differ in terms of histochemical properties (Gollnick et al. 1974c, Edgerton et al. 1975) Ochs et al. (1977) observed a more pronounced increase in E/T ratio of the gastrocnemii muscle during repeated plantar flexions. EMG studies of human dorsal interossei muscles have shown that FT motor units fatigue more easily than ST units. In addition a broader range in fatigability was observed for FT motor units as compared to ST motor units (Stephens & Dechenwood 1975). Decrease in the EMG activity during maximal effort is suggested to be due to inhibition of some motor units and/or changes in the form of motor unit potential e.g. amplitude, duration, frequency spectrum. Support for the latter possibility was demonstrated in experiments leading to conditions referred to as ischaemic (Mortimer et al. 1970). In agreement with human experiments (Stålberg 1966) a decrease in the conduction velocity of stimulated cat muscle fibres was observed, which was suggested to account for observed changes in the EMG frequency spectrum. The decrease in conduction velocity was more pronounced in the gastrocnemii as compared to the soleus muscle. The authors suggested lactate as responsible for these changes although no data were obtained on lactate concentration.

## Lactate accumulation and muscle fatigue

One of the basic findings in the present study was the higher rate of lactate formation observed in FT as compared to ST fibres. The results agree with the data obtained in animal experiments (Ogata 1960 Beatty et al. 1963) and previously suggested by Fletcher (1914). A lactate "flow" from FT to ST fibres may have occurred as discussed by Essén et al. (1975b) mediated by membrane bound M-LDH as suggested by Sjödin (1976) and thus partly explaining the lactate content in ST fibres.

Concomitant with a reduction in ATP and CP stores there is an increased lactate formation (Bultman et al. 1967 Karlsson 1971). Lactate accumulation depends on lactate production and release. As demonstrated lactate release in man is found to be maximal already at muscle lactate concentrations corresponding to approximately 5 mmol  $\times$  kg<sup>-1</sup> wet muscle (Jocfeldt et al. 1978). The authors did not indicate whether the assumed translocation hindrances for lactate were related to individual muscle fibre type distribution. Following submaximal cycling exercise greater muscle/blood lactate gradients were observed in individuals with muscles rich in FT fibres as compared to what was found in individuals with muscles rich in ST fibres (Graham et al. 1978). Accordingly the present results suggest that individuals with muscles rich in high oxidative (ST and/or Fth) fibres have a greater release most probably due to a more developed capillary network (Andersen 1975 Andersen & Henriksen 1977). This suggestion is in agreement with the findings by Folkow & Halicka (1968) comparing "red" and "white" muscles of cat with respect to blood supply, capillary surface area and oxygen uptake at rest and during exercise.

A causal relationship between muscle fatigue and lactate accumulation has been suggested (Assmusen et al. 1948 Karlsson 1971 Kleusen et al. 1972). Support for this view has been found in human experiments where performance capacity of longer duration (3-5 min) was found to be negatively affected by elevated blood and muscle lactate levels induced by previous exercise of other muscles (Karlsson et al. 1975). Accordingly the same lactate concentrations were demonstrated during maximal exercise at various ambient pressures concomitant to increased performance time with increased ambient pressure (Linnarsson et al. 1974).

In muscle biopsy specimens a close relationship between pH and lactate concentration immediately at termination of exercise has been demonstrated (Sahlin 1978) and the same muscle vein pH was demonstrated on exhaustion during maximal exercise at varying ambient pressures (Kajzer 1970) indicating that increased acidity in the muscle may determine the point of exhaustion. The mechanism for lactate fatigue is however not clear. A mechanism for high H<sup>+</sup> concentrations to cause impaired muscle function has been demonstrated by Fuchs et al. (1970) and Malomura & Schwarz (1972). They showed that an increase in the H<sup>+</sup> concentration decreases the number of calcium ions bound to troponin during the excitation-contraction coupling. This would reduce the number of active actin-myosin interactions thus decreasing contractile force. Experiments on skinned frog muscle fibres have supported this hypothesis (Robertson & Kerrick 1979). Thus intracellular acidosis depressed mechanical function at the

actomyosin interaction level. In addition a lowered pH was observed to require a higher  $\text{Ca}^{2+}$  concentration to result in a given level of activation. Eberstein & Sendow (1963) have published evidence indicating that the functional site of fatigue is in the link that couples excitation to contraction either reducing the intensity of the excitation-contraction link or decreasing the sensitivity to activation. Changes in flux of calcium were suggested to alter the operation of excitation-contraction link.

Recently Pitts & Balloney (1976-1978) were able to demonstrate a linear relationship between twitch and tetanic tension and lactate concentration of stimulated frog sartorius muscle. During 30 s of recovery however contractile twitch tension and ATP concentration increased rapidly while no significant reduction in lactate concentration occurred during this period. In experiments where cat gastrocnemii and soleus muscles were stimulated to contract a discrepancy in fatigue pattern was demonstrated between muscles in accordance with Kugelberg & Edström (1968) Hamnerberg & Kallert (1975). A 10 s pause in stimulation was found to result in a complete recovery in contractile strength of the gastrocnemii muscle. The soleus muscle on the other hand displayed a less pronounced recovery from fatigue. A more rapid restitution of ATP due to higher activity of creatine phosphokinase (Bucke & Edgerton 1975) in the fast contracting gastrocnemii muscle was suggested to explain in part the discrepancy in recovery pattern. In preliminary experiments using the fatigue test described here a rapid restitution of contractile tension was observed during the first 15 s of recovery from exercise in spite of unchanged or slightly decreased lactate levels. Due to limited number of subjects studied the influence of fibre type distribution could not be elucidated.

#### Central versus peripheral fatigue

"Muscle fatigue" is a complex phenomenon with numerous possible sites of origin. "Central fatigue" in contrast to "peripheral fatigue" was suggested already in 1903 by Setchenov and even though Bigland-Ritchie et al (1978) calculated that "central fatigue" may account for 30% of total force loss in sustained isometric contractions of the human quadriceps muscle the phenomenon was not observed during the initial phase of contraction. Hence force produced through voluntary contractions and contractions stimulated via the femoral nerve decreased to a similar extent during the first 30 s of contraction. One cannot preclude that changes in the "milieu interne" of the activated muscle via sensory receptors and their afferents will result in a central inhibition as has been suggested (e.g. Reid 1928, Lippold et al 1960, Amussen & Maxin 1978a). When again considering experiments originally described by Setchenov (1903) Amussen & Maxin (1978a, b) it was demonstrated that exhausted individuals in a second bout of exercise were able to perform more work if diverting mental or physical activity were introduced in the pause instead of only rest. The observation was considered to be due to a change in the balance between the outflow

of inhibitory and facilitatory impulses to the central nervous system implicating that central inhibition might influence mechanical output during voluntary contraction

Whether or not central fatigue occurs failure at or distal to the neuromuscular junction must be considered mainly to limit the extent of voluntary contraction. Thus muscle contraction impairment may arise from failure of transmission at the neuromuscular junction action potentials which fail to propagate or from disturbances in calcium uptake release or binding capacity due to internal metabolic changes concomitantly affecting the coupling processes

#### SUMMARY

- 1 Physically active young men were studied in terms of muscle fibre type distribution in the vastus lateralis muscle and performance capacity during various types of short term exercise. The percentage of fast twitch (FT) muscle fibres averaged 49 (range 10-79) % in the studied muscle
- 2 Muscle force (torque) was recorded during repeated isokinetic knee extensions. The absolute as well as relative torque decline was taken as criterion for muscle fatigue and was found to be more pronounced in individuals with muscles rich in FT and/or FTb muscle fibres
- 3 Electromyography (EMG) was recorded from the vastus lateralis using surface electrodes. It was analyzed for integrated EMG (IEMG) and power spectral density function (PSDF). In contrast to IEMG a general decrease in mean power frequency (MPF) was observed during repeated muscle contractions. This shift was due to a decrease in the high frequency bandwidth (associated with FT motor units) of EMG with a simultaneous increase in the bandwidth of low frequency components. In individuals characterized by a high percentage of FT fibres MPF decreased to a higher extent as compared to individuals with a high percentage of ST fibres
- 4 Lactate concentration and the activity of LDH<sub>tot</sub> and M-LDH were analysed in pools of different muscle fibre types. After various types of exercise (25 isokinetic contractions, supramaximal cycling exercise, downhill skiing) lactate concentration was related to % FT fibres in the activated muscle. Lactate concentration was also related to LDH<sub>tot</sub> and M-LDH activity. This seems to indicate a higher rate of lactate production in FT fibres as compared to ST fibres
- 5 After 25 maximal repeated contractions a relationship between force decline and FT/ST lactate ratio was demonstrated. A similar relationship was also demonstrated for LDH<sub>tot</sub> and M-LDH ratios indicating a close relationship between the metabolic profile of the different fibre types, muscle metabolism and muscle function

- || Lactate accumulation and associated metabolic changes interfering with the contractile mechanism is suggested to be responsible for muscle fatigue in the situations described here

#### ACKNOWLEDGEMENTS

This work was carried out at the Department of Physiology III at Gynastik- och idrottshögskolan, Karolinska Institute Stockholm, directed by Professor Per-Olof Astrom, and at the Laboratory for Human Performance National Defence Research Institute Stockholm, directed by Professor Charles B Strömblad

I wish to express my gratitude to Dr Jan Karlsson who introduced me to the field of exercise physiology for guidance and enthusiastic support throughout the investigation. I am also indebted to Professor Lennart Kaijser and Dr Bertil Sjödin for valuable advice and constructive criticisms.

My sincere thanks also to Miss Gurlieth Petersson for excellent and patient secretarial help to Mrs Karin Elvin and Mrs Berit Sjöberg for skilful technical assistance and to my colleagues and co-workers who contributed to the stimulating atmosphere

I wish to express my appreciation to all volunteers who participated in the studies

This investigation was supported by grants from the Swedish Medical Research Council (No 4251) and from the Research Council of the Swedish Sports Federation.

## REFERENCES

- ANDERSEN H 1975 Capillary density in skeletal muscle of man. *Acta Physiol Scand* 95:203-205
- ANDERSEN P & BJØGAARD G 1976 Selective glycogen depletion in the subgroups of type II muscle fibres during intense submaximal exercise in man. *Acta Physiol Scand* 96:26A.
- ANDERSEN H & BERGSTRÖM J 1977 Capillary supply of the quadriceps femoris muscle of man: adaptive responses to exercise. *J Physiol* 270:677-690
- ASMUSSEN E, DÖBELN von W & HEDLÉN M. 1948 Blood lactate and oxygen debt after exhaustive work at different oxygen tensions. *Acta Physiol Scand* 15 57-62
- ASMUSSEN E. & MAXIN B 1978a. Recuperation after muscular fatigue by "Diverting Activities". *Eur J Appl Physiol* 38:1-7
- ASMUSSEN E. & MAXIN B 1978b. A central nervous component in local muscular fatigue. *Eur J Appl Physiol* 38:9-15
- ASTRAND P-O & SALTEIN B 1961 Oxygen uptake during the first minutes of heavy muscular exercise. *J Appl Physiol* 16:971-976
- BAUDIN K M. & TIPTON C.M. 1972 Work and metabolic patterns of fast and slow twitch skeletal muscle contracting in situ. *Pflügers Arch* 334:345-356
- BANG H 1936 The lactate content of the blood during and after muscular exercise in man. *Scand Archiv für Physiologie Suppl* 10 74:51-82
- BARANY M. 1967 ATPase activity of myosin correlated with speed of muscle shortening. *J gen Physiol* 50:197-218
- BARANY M. & CLOSE R.I 1971 The transformation of myosin in cross-innervated rat muscles. *J Physiol (Lond.)* 213:455-474
- BARTLEY S. & CHUTE E 1947 Fatigue and impairment in man. McGraw-Hill Book Company Inc. New York.
- BARTLEY S R. 1965 Fatigue: Mechanism and management. Charles C Thomas Publisher Springfield, Illinois
- BEATTY C.H., PETERSON R.D. & BOCK, R.M. 1963 Metabolism of red and white muscle fiber groups. *Am J Physiol* 204(5) 939-942
- BENDAT J S & PIERSON, A.G 1971 Random data Analysis and measurement procedures John Wiley & Sons New York
- BERGSTRÖM, J 1962 Muscle electrolytes in man. *Scand J clin Lab Invest. Suppl* 68
- BERGSTRÖM, J, HARRIS R.C, HOLMANN E. & NORDSTED L.-O 1971 Energy rich phosphagens in dynamic and static work. *Adv Exp Med Biol* 11:341-355

- HIGLAND-RITCHIE B & WOODS J.J 1974 Integrated EMG and oxygen uptake during dynamic contractions of human muscles *J Appl Physiol* 36 475-479
- HIGLAND-RITCHIE, B JONES D.A. HOSKING G.P & EDWARDS R.H T 1978 Central and peripheral fatigue in sustained maximum voluntary contractions of human quadriceps muscle *Clin Sci Mol Med* 54:609-614
- HOLSTAD G. & ERSLAND A. 1978 Energy metabolism in different human skeletal muscles during voluntary isometric contractions *Eur J Appl Physiol* 38:171-179
- HODAL, P INHATER, F & HERNANSEN L. 1977 Capillary supply of skeletal muscle fibers in untrained and endurance-trained men *Am J Physiol* 232(6):H705-H712
- BROOKE, M.H & KAIBER, K.K 1970 Three "myosin adenosine triphosphatase" systems: The nature of their pH lability and sulfhydryl dependence *J Histochem Cytochem* 18 670-672
- BROWN G.L. & BURNS B.D. 1949 Fatigue and neuromuscular block in mammalian skeletal muscle *Proc Roy Soc B (Lond)* 136:182-195
- BURKE R.E. LEVINE, D.H EATON P.E. TSARIS P & EGEL W.K. 1971 Mammalian motor units: Physiological-histochemical correlation in three types in cat gastrocnemius *Science* N.Y. 174 709-712
- BURKE, R.E & EDERSON V.R. 1975 Motor unit properties and selective involvement in movement. In: *Exercise and sport sciences reviews* pp 31-61 Vol. 3 Academic Press New York.
- CLOSE, R.I. 1972 Dynamic properties of mammalian skeletal muscles *Physiological Reviews* 52:129-197
- COBB S & FORBES A. 1923 Electromyographic studies of muscular fatigue in man *Am J Physiol* 88 234-251
- COYLE, E.F COSTILL, D.L. & LEMMONS G.R. 1979 Leg extension power and muscle fiber composition. *Med Sci Sports* 11 12-15
- DANFORTH W.H. 1965 Activation of glycolytic pathway in muscle. In: *Control of energy metabolism* (ed. B. Chance et al.) pp 287-297 Academic Press New York
- DIETZ A.A. & LUBSKY, T. 1967 Separation and quantitation of lactate dehydrogenase isoenzymes by disc electrophoresis *Analyt Biochem* 20 246-257
- DONOFRIO J. 1961 The metabolism of the tonic and tetanic muscles. I. Glycolytic metabolism. *Arch Biochem and Biophysics* 95 138-143
- EBERSTEIN A. & SANDOW A. 1961 Fatigue in phasic and tonic fibers of frog muscle *Science* 11 283-284
- EBERSTEIN A. & SANDOW A. 1963 Fatigue mechanisms in muscle fibres. In: *The effect of use and disuse of neuromuscular functions* (ed E. Guberman and P. Huk) pp. 515-526 Elsevier Publishing Company Amsterdam.



- EDGERTON V R SMITH J L & SIMPSON D R. 1975 Muscle fibre type populations of human leg muscles. *Histochemical Journal* 7:259-266
- EDSTRÖM, L & EGELON B 1972 Differences in sizes of red and white muscle fibres in vastus lateralis of musculus quadriceps femoris of normal individuals and athletes. Relation to physical performance. *Scand J clin Lab Invest* 30:175-181
- EDWARDS R.G & LIPPOLD O C.J 1956 The relation between force and integrated electrical activity in fatigued muscle. *J Physiol* 132:677-681
- ENGEL W.K. 1962 The essentiality of histo- and cytochemical studies of skeletal muscle in the investigation of neuromuscular disease. *Neurology (Minneapolis)* 12:778-794
- ERIKSSON A. ENGBOM, J. HULTEN B. KARLSSON E & KARLSSON J 1976 Anatomical, histological and physiological factors in experienced downhill skiers. *Orthopedic Clinics of North America* 7:159-165
- ESSEN B & HÄGGMARK T 1975 Lactate concentration in type I and II muscle fibres during muscular contraction in men. *Acta Physiol Scand* 95:344-346
- ESSEN B, JAKSSON E, HENRIKSSON J, TAYLOR A.W & SALTIN B 1975a Metabolic characteristics of fibre types in human skeletal muscle. *Acta Physiol Scand* 95: 153-165
- ESSEN B, PERSSON B, GOLLIGRICK, P D & SALTIN B 1975b Muscle glycogen content and lactate uptake in exercising muscles. In: *Metabolic adaptation to prolonged physical exercise* (ed. H. Rowell and J.R. Poortmans) pp 130-134. *Birchhäuser Verlag*, Basel.
- ESSEN B. 1978 Studies on the regulation of metabolism in human skeletal muscle using intermittent exercise as an experimental model. *Acta Physiol Scand Suppl* 454
- FITTS R.H. & HOLLOSZY J O 1976 Lactate and contractile force in frog muscle during development of fatigue and recovery. *Am J Physiol* 231(2):430-433
- FITTS R.H. & HOLLOSZY J O 1977 Contractile properties of rat soleus muscle: effects of training and fatigue. *Am J Physiol* 233(3):C86-C91
- FITTS R.H. & HOLLOSZY J O 1978 Effects of fatigue and recovery on contractile properties of frog muscle. *J Appl Physiol:Respirat Environ Exercise Physiol* 45(6) 899-902
- FLETCHER, W.M. & HOPKINS F G 1907 Lactic acid in amphibian muscle. *J Physiol (Lond)* 35:247-250
- FLETCHER, W.M. 1914 Lactic acid formation: survival respiration and rigor mortis in mammalian muscle. *J Physiol* 47 361-380
- FOLKOW H & HALICKA, H.D 1968 A comparison between "red" and "white" muscle with respect to blood supply, capillary surface area and oxygen uptake during rest and exercise. *Microvascular Research* 1 1-14

- FOCHE M REDDY Y & BRIGGS F N 1970 The interaction of cations with the calcium-binding site of troponin. *Biochim Biophys Acta* 221:407-409
- GOLDSPIK, G LARSON R.E & DAVIES R.E 1970 The immediate energy supply and the cost of maintenance of isometric tension for different muscles in the hamster. *J. vargl Physiol* 66 389-397
- GOLLIK, P D PIEHL, K. SHERBERT IV C W ARMSTRONG R.B & SALTIN B 1972a. Diet exercise and glycogen changes in human muscle fibers. *J Appl Physiol* 33(4) 421-425
- GOLLIK, P D ARMSTRONG R.B SHERBERT IV C W PIEHL, K. & SALTIN B 1972b Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. *J Appl Physiol* 33(3):312-319
- GOLLIK, P D ARMSTRONG R.B SHERBONICH W.L. SHEPHERD R.E & SALTIN B 1973a Glycogen depletion pattern in human skeletal muscle fibers after heavy exercise. *J Appl Physiol* 34(5) 615-618
- GOLLIK, P.D ARMSTRONG R.B SHERBERT IV C W SHERBONICH W.L. SHEPHERD R.E. & SALTIN B. 1973b. Glycogen depletion patterns in human skeletal muscle fibers during prolonged work. *Pflügers Arch* 344:1-12
- GOLLIK, P D PIEHL K. & SALTIN B 1974a. Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J Physiol (Lond.)* 241:45-57
- GOLLIK, P D KARLSSON J PIEHL K & SALTIN B. 1974b Selective glycogen depletion in skeletal muscle fibres of man following sustained contractions. *J Physiol (Lond.)* 241:59-67
- GOLLIK, P.D SUTIN B KARLSSON J JANSSON E. & SALTIN B 1974c Human soleus muscle. A comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Arch* 348:247-255
- GOMORI, G. 1941 The distribution of phosphatase in normal organs and tissues. *J Cell Comp Physiol* 17:71-83
- GRAHAM, T BJØGAARD G. LILJGREN H. & SALTIN B. 1978 NAD in muscle of man at rest and during exercise. *Pflügers Arch* 376 35-39
- GRIMBY L. & HANSSON J 1977 Firing rate and recruitment order of toe extensor motor units in different modes of voluntary contraction. *J Physiol (Lond )* 264 865-879
- GUDIMOV A. & KOSAROV D 1974 Some features of different motor units in human biceps brachii. *Pflügers Arch* 347:75-88
- HOGGARK, T JANSSON E & SVANE B 1978 Cross-sectional area of the thigh muscle in man measured by computed tomography. *Scand J Clin Lab Invest* 38 353-360
- HANSSONBERG, C. & KELLERKIH J-O 1975 Studies of some twitch and fatigue properties of different motor unit types in the ankle muscles of the adult cat. *Acta Physiol Scand* 95 231-242

- EDGERTON V.R., SMITH J.L. & SIMPSON D.R. 1975 Muscle fibre type populations of human leg muscles. *Histochemical Journal* 7:259-266
- EDSTRÖM, L. & EKELOM, B. 1972 Differences in sizes of red and white muscle fibres in vastus lateralis of musculus quadriceps femoris of normal individuals and athletes. Relation to physical performance. *Scand J clin Lab Invest* 30:175-181
- EDWARDS E.G. & LIPPOLD O.C.J. 1956 The relation between force and integrated electrical activity in fatigued muscle. *J Physiol* 132:677-681
- ENGEL W.K. 1962 The essentiality of histo- and cytochemical studies of skeletal muscle in the investigation of neuromuscular disease. *Neurology (Minneapolis)* 12:778-794
- ERIKSSON A., ERIKSSON, J., EKLUND, B., KARLSSON E. & KARLSSON J. 1976 Anatomical, histological and physiological factors in experienced downhill skiers. *Orthopaedic Clinics of North America* 7:159-165
- ESSEN B. & HOGMARK T. 1975 Lactate concentration in type I and II muscle fibres during muscular contraction in man. *Acta Physiol Scand* 95:344-346
- ESSEN B., JONSSON E., HENRIKSSON J., TAYLOR, A.W. & SALTIN B. 1975a Metabolic characteristics of fibre types in human skeletal muscle. *Acta Physiol Scand* 95: 153-165
- ESSEN B., PERSSON B., GÖLLRICK P.D. & SALTIN B. 1975b Muscle glycogen content and lactate uptake in exercising muscles. In: *Metabolic adaptation to prolonged physical exercise* (ed. H. Rowell and J.R. Poortmans) pp 130-134. *Birkhäuser Verlag*, Basel.
- ESSEN B. 1978 Studies on the regulation of metabolism in human skeletal muscle using intermittent exercise as an experimental model. *Acta Physiol Scand Suppl* 454
- FITTS R.H. & HOLLOSZY J.O. 1976 Lactate and contractile force in frog muscle during development of fatigue and recovery. *Am J Physiol* 231(2):430-433
- FITTS R.H. & HOLLOSZY J.O. 1977 Contractile properties of rat soleus muscle: effects of training and fatigue. *Am J Physiol* 233(3):C86-C91
- FITTS R.H. & HOLLOSZY J.O. 1978 Effects of fatigue and recovery on contractile properties of frog muscle. *J Appl Physiol:Respirat Environ Exercise Physiol* 45(6): 899-902
- FLETCHER, W.M. & HOPKINS P.G. 1907 Lactic acid in amphibian muscle. *J Physiol (Lond)* 35:247-250
- FLETCHER, W.M. 1914 Lactic acid formation, survival respiration and rigor mortis in mammalian muscle. *J Physiol* 47: 361-380
- FOLKOW B. & HALICAKA, H.B. 1968 A comparison between red and "white" muscles with respect to blood supply, capillary surface area and oxygen uptake during rest and exercise. *Microvascular Research* 1: 1-14

- KALLNER, L. 1970 Limiting factors for aerobic muscle performance. *Acta Physiol Scand*, Suppl. 346
- KARLSSON J. 1971 Lactate and phosphagen concentrations in working muscle of man. *Acta Physiol Scand*, Suppl. 358
- KARLSSON J., FRITH, K., SJÖDIN B., GOLINICK P.D. & SALTIN B. 1974 Distribution of LDH isoenzymes in human skeletal muscle. *Scand J clin Lab Invest* 33:307-312
- KARLSSON J., BORDE-PETERSEN F., BENNICKSSON J. & KNUTZEN H.H. 1975 Effects of previous exercise with arms or legs on metabolism and performance in exhaustive exercise. *J Appl Physiol* 38:763-767
- KINDEN K., KNUTZEN H.G. & POSTER, H.V. 1972 Effect of pre-existing high blood lactate concentration on maximal exercise performance. *Scand J clin Lab Invest* 30 415-419
- KOGI K. & HAKAWADA, T. 1962 Slowing of surface electromyogram and muscle strength in muscle fatigue. *Rep Inst Sc Lab* 60:27-41
- KOMI P.V. & LESTIÖ P.K. 1973 A computerized data processing system for a fatigue experiment with a special reference to EMG activity. *Proc First National Meeting on Biophysics and Biotechnology* Helsinki, Technical Research Centre of Finland pp 211-213
- KOMI P.V. & VIITASALO J.H.T. 1976 Signal characteristics of EMG at different levels of muscle tension. *Acta Physiol Scand* 96 267-276
- KUGLBERG E. & EDSTRÖM, L. 1968 Differential histochemical effects of muscle contractions on phosphorylase and glycogen in various types of fibres: relation to fatigue. *J Neurol Neurosurg Psychiat* 31:415-423
- KUSUDA, E., KLISOURAS V. & MILSON, J.H. 1970 Electrical and metabolic activities and fatigue in human isometric contraction. *J Appl Physiol* 29 358-367
- LARSSON L. 1978 Morphological and functional characteristics of the ageing skeletal muscle in man. A cross-sectional study. *Acta Physiol Scand* Suppl. 457
- LEE, F.S. 1906 Fatigue. *Harvey lectures* 1:169-194
- LESHER G.R., HEYMAN, D.W., COSTILL, D.L. & FINK W.J. 1979 Glycogen depletion in skeletal muscle during maximal isokinetic exercise. *Med Sci Sports* 11(1) 113
- LINDBSTRÖM, L., MAGNUSSEN R. & PETERSEN I. 1970 Muscular fatigue and action potential conduction velocity changes studied with frequency analysis of EMG signals. *Electromyography* 4 341-356
- LINDBERSSON D., KARLSSON, J., FAGRAHUS L. & SALTIN B. 1974 Muscle metabolites and oxygen deficit with exercise in hypoxia and hyperoxia. *J Appl Physiol* 36:399-402
- LIPPOLD O.C.J. 1955 Fatigue of finger muscles. *J Physiol* 128:33P
- LIPPOLD, O.C.J., REDEZANI J.W.T. & VUOLO J. 1960 The electromyography of fatigue. *Ergonomics* 3 127-131

- SEICHENOV I.M. 1935 Zur Frage nach der Eindringung sensitiver Reize auf die Muskelarbeit des Menschen. In: Selected works pp 246-260 Moscow
- SIMONSON E 1971 Physiology of work capacity and fatigue Charles C Thomas Springfield Illinois
- SJÖDIN B 1976 Lactate dehydrogenase in human skeletal muscle Acta Physiol Scand, Suppl. 436
- SPARKE J I & SCHOTTELLUS B.A. 1970 Chemical basis of fatigue in isolated mouse soleus muscle. Am J Physiol 219(5):1490-1495
- STÅLBERG E 1966 Propagation velocity in human muscle fibers in situ. Acta Physiol Scand, Suppl. 287
- STEPHENS J.A. & TAYLOR, A. 1972 Fatigue of maintained voluntary muscle contraction in man. J Physiol (Lond.) 220:1-18
- STEPHENS J.A. & USHERWOOD T P 1975 The fatigability of human motor units J Physiol 250:37-38P
- STRÖM, G 1949 The influence of anaemia on lactate utilisation in man after prolonged muscular work Acta Physiol Scand 17:440-451
- TESEH P & KARLSSON J 1977 Lactate in fast and slow twitch skeletal muscle fibres of man during isometric contraction Acta Physiol Scand 99:230-236
- TRUSTILE H.G. HESLOP H.J. MOFFROID M. & LONGMAN E W 1967 Isokinetic contraction: a new concept of resistive exercise Arch Phys Med Rehab 48:279-282
- THOMSON J.A. GREEN H J & HOUSTON M.E. 1979 Muscle glycogen depletion patterns in fast twitch fibre subgroups of man during submaximal and supramaximal exercise Pflügers Arch 379:105-108
- THORSTENSSON A. 1976 Muscle strength fibre types and enzyme activities in man Acta Physiol Scand, Suppl. 443
- THORSTENSSON A. & KARLSSON J 1976 Fatigability and fibre composition of human skeletal muscle Acta Physiol Scand 96:318-322
- THORSTENSSON A. SJÖDIN B TESEH, P & KARLSSON J 1977 Actomyosin ATPase myo-kinase CPK and LDH in human fast and slow twitch muscle fibres Acta Physiol Scand 99:225-229
- WACHOLDER, K. 1931 Weitere Untersuchungen über das Verhalten "tonischer" und "nicht tonischer" Muskeln bei ermüdender Reizung Pflügers Arch 229:133-142
- WARMOLIS J R. & ENGEL W K. 1972 Open biopsy electromyography Arch Neurol (Chic ) 27:512-517
- VITASALO J B.T & KOMI P V 1975 Signal characteristics of EMG with special reference to reproducibility of measurements Acta Physiol Scand 93:531-539
- VITASALO J B.T & KOMI P V 1977 Signal characteristics of EMG during fatigue Eur J Appl Physiol 37 111-121





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 480

**Muscle fatigue in man**  
with special reference to lactate accumulation  
during short term intense exercise

By  
PER TESCH





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 480

From the Department of Clinical Physiology Karolinska sjukhuset and  
Department of Physiology III Karolinska institutet Stockholm, Sweden

# Muscle fatigue in man

with special reference to lactate accumulation  
during short term intense exercise

By  
PER TESCH

STOCKHOLM 1980

The present thesis is based on the following papers which will be referred to by their Roman numerals

- I Teuch, P B Sjödin and J Karlsson Relationship between lactate accumulation LDH activity LDH isoenzyme and fibre type distribution in human skeletal muscle Acta physiol scand 1978 103 40-46
- II Teuch P B Sjödin A. Thorstensson and J Karlsson Muscle fatigue and its relation to lactate accumulation and LDH activity in man Acta physiol scand. 1978 103 413-420
- III Nilsson, J P Teuch and A. Thorstensson Fatigue and EMG of repeated fast voluntary contractions in man Acta physiol scand 1977 101 194-198
- IV Komi, P V and P Teuch. EMG frequency spectrum muscle structure and fatigue during dynamic contractions in man. Eur J Appl Physiol 1979 42 41-50
- V Teuch P Fatigue pattern in subtypes of human skeletal muscle fibres Int J Sports Med Accepted for publication
- VI Teuch P Local lactate and exhaustion Acta physiol scand 1978 104 373-374
- VII Teuch P L. Larsson A. Eriksson and J Karlsson Muscle glycogen depletion and lactate concentration during downhill skiing Med Sci Sports 1978 10 85-90

In addition some unpublished results are included.

## INTRODUCTION

The feeling of discomfort and pain due to heavy muscular exercise is an experience with which most individuals are familiar. Housework, occupational work, leisure as well as sport activities will sometimes lead to these states which can be referred to as fatigue experience. A conclusive definition of the fatigue phenomenon does not exist (cf Bartley & Chute 1947, Bartley 1965, Simonson 1971) although the question of its origin has been the subject of controversy among researchers in various scientific fields during more than half a century. Monro (1890) who constructed and used a finger ergograph was one of the first to report changes in mechanical performance during repeated muscle contractions. Since then muscular fatigue as indicated by a failure to generate maximum voluntary force or a prescribed force level has been extensively studied in order to establish its possible sites of origin (e.g. Lee 1906, Cobb & Forbes 1923, Reid 1928, Brown & Burns 1949, Marton 1954, Neess & Stone-Mathisen 1955, Scherrer & Bourguignon 1959, Lippold et al. 1960, Stephens & Taylor 1972, Bigland-Ritchie et al. 1978).

Interest has also been focused on the behaviour of different motor unit types including their extrafusal muscle fibres in terms of fatigability. Recent review articles concerning specific characteristics of different motor units in animal (Close 1972, Burke & Edgerton 1975) as well as man (Saltin et al. 1977) are available. As already reviewed by Needham (1926) the classical "red" muscle possesses a greater resistance to fatigue as compared to the "white" muscle. By means of mechanical definition of motor units, Weickholder (1931) demonstrated that tonic muscles are more fatigue resistant as compared to phasic muscles. Later this pattern has been confirmed in a number of animal studies (e.g. Ehrenstein & Sandow 1961, 1963, Riegelberg & Edström 1968, Burke et al. 1971). In fact the property in this respect has been included in the fibre type nomenclature (cf Burke & Edgerton 1975) in order to distinguish between fast twitch (FT) muscle fibres with different fatigability levels.

Recently it has been possible also in man to relate sustained (Milbech & Johansen 1973, Ruitén et al. 1975) and repeated (Ochs et al. 1977) isometric contraction fatigue directly or indirectly to the histochemical profile of the activated muscle. Using a device to study dynamic contractions (Parrine 1968) based on the force-velocity principle as introduced by Hill (1938), Thorstensson (1976) was able to demonstrate a positive relationship between the individual percentage of fast twitch (type II) muscle fibres in the vastus lateralis muscle and the decrease in force output during repeated knee extensions.

Attempts have been made to explain the basic features of fatigue by relating metabolic and mechanical changes which occur during exercise (Hill & Riegelberg 1930). Since the work of Fletcher & Hopkins (1907) many authors have suggested lactate accumulation as directly or indirectly causing an impaired muscular function (e.g. Bang 1936, Amundsen et al. 1948, Karlsson 1971, Karlsson et al. 1975). Some electrophysiologists who have studied changes in action potential conduction velocity

and the frequency patterns of motor units agree on this suggestion (e.g. Lindström et al. 1970, Mortimer et al. 1970). Other experimental observations however speak in favour of a block at the neuromuscular junction (Stephens & Taylor 1972) as the site of muscle fatigue.

It is generally believed that at the onset of heavy muscular activities adenosine triphosphate (ATP) and creatine phosphate (CP) stored in the muscle exclusively will cover the energy demand. Theoretically however these immediate energy sources can be depleted in man within 10 seconds (Margaria et al. 1964). Such short periods of maximal exercise concomitantly result in the formation of lactate (Pezow & Mehren 1962, Bultman et al. 1967, Bergström et al. 1971, Saltin et al. 1971). Simultaneously signs of muscle fatigue or impairment can be recognized as indicated by a decrease in mechanical output within this time limit. In order to elucidate the fatigue phenomenon, the present study was designed by combining established histochemical, biochemical and electrophysiological methods when examining high intensity exercise of short duration. More specifically experiments were carried out:

- a. To study physically active males with muscles differing in muscle fibre type distribution with regard to performance capacity during short term dynamic exercise of high intensity
- b. To analyse lactate concentration in pools of different muscle fibre types following short term intense exercise
- c. To study electromyographic (EMG) patterns with emphasis on changes in frequency spectrum of motor units during exercise as described above

#### SUBJECTS

A total of 64 subjects was studied. Age, height and weight averaged 24 (range 19-42) yrs, 180 (range 170-194) cm, and 72 (range 60-89) kg. They were all physically active and most were physical education students or subjects with a similar background. Among these were 14 individuals participating in more regular physical training programs and competing on the national elite level in cycling, long-distance running, orienteering and downhill skiing. In certain cases subjects were involved in more than one experiment. Major physical characteristics of the subjects are summarized in Table 1. Prior to giving their oral consent to participate in the study, the subjects were informed of the purpose and the risks associated with the testing procedures. The investigation was approved by the Ethical Committee at Karolinska Institutet, Stockholm.

Table 1 Characteristics of subject groups. Mean (SD) values or range are given. Maximal oxygen uptake was determined using exercise on a cycle ergometer

Study	n	Age yrs	Height cm	Weight kg	$\dot{V}O_2$ max ml $\cdot$ kg <sup>-1</sup> $\times$ min <sup>-1</sup>	Category
I	10	24-1	179-1	71 3 <sup>±</sup> 2 1	59 1-1 9	Physical education students
II	9	23-1	179-1	69 9 <sup>±</sup> 1 8	60 5-1 4	—
III	12	22 <sup>±</sup> 1	180 <sup>±</sup> 1	70 3-1 6	—	Physical education students Endurance trained
IV	11	25-2	179-1	72 0 <sup>±</sup> 1 9	59 1 <sup>±</sup> 1 8	Physical education students
V	23	24 <sup>±</sup> 1	180 <sup>±</sup> 1	72 2 <sup>±</sup> 1 7	—	Physical education students Endurance trained
VI	7	22-1	180 <sup>±</sup> 1	69 3-2 0	65 5-1 3	Endurance trained
VII	8	24 <sup>±</sup> 1	178-1	69 9 <sup>±</sup> 1 8	—	Physical education students
	3	20-22	174 182	72-76	—	Downhill skiers
	2	28-42	170-177	66-75	—	Ski school instructors
	8	24-1	181 <sup>±</sup> 1	74 2-1 5	—	Physical education students

### Statistics

Ordinary statistical methods were used to calculate mean SE and linear correlation coefficient ( $r$ ). When useful intra- and interindividual differences and differences between means were tested for significance using student's  $t$  test. Calculation of error of methods was conducted on duplicate analyses and expressed as the coefficient of variation ( $C.V. = SD$  of the difference between double values  $\times \frac{1}{\sqrt{2}} \times \frac{100}{\bar{x}}$ )

### Force measurement (I-V)

In order to examine the early onset of muscle fatigue force (torque) development during repeated knee extensions was recorded using isokinetic equipment (Cyber II Lumex Inc. New York). The principles of the apparatus have been described in detail elsewhere (e.g. Hsiao & Perrine 1967, Thistle et al. 1967). The reliability and validity of the apparatus are high as reported by Moffroid et al. (1969) and Thorstenson (1976). The subjects were firmly fixed in a sitting position in a specifically constructed chair. The lever arm of the dynamometer length held constant was attached to the lower left leg of the subjects. (For further information concerning experimental set-up see Thorstenson 1976). Torque was measured at pre-set constant angular velocities corresponding to  $180$  and  $300^\circ \times s^{-1}$  ( $\pi$  and  $5\pi/3$  rad  $s^{-1}$ ) respectively.

Peak torque of the total torque curve reproduced by a UV-recorder was measured. The curve represented a motion range from  $100^\circ$  ( $0.55\pi$ ) to fully extended knee joint ( $0^\circ$ ). Work was determined as the area under the torque curve and measured with a planimetric technique from the recording paper. Power was calculated as work divided by the contraction time.

The individual maximal peak torque (MPT) was defined as the highest value attained from two single attempts with 30-40 s of recovery in between. Coefficient of variation ( $c.v.$ ) when comparing torque developed in two consecutive attempts was 2.1% at  $180^\circ \times s^{-1}$  as compared to 8.0% at  $300^\circ \times s^{-1}$  ( $n = 18$ ).

When studying muscle fatigue maximal knee extensions at  $180^\circ \times s^{-1}$  were repeated 25, 50 and 100 times respectively. Each contraction phase lasted 0.5 s and the following recovery period approximately 7 s. Thus a complete contraction cycle corresponded to 1.2 s. No systematic variations were observed in the time variables during one single experiment or between experiments. Performance times for the fatigue experiments were then 30, 60 and 120 s. Experiments comprising 100 contractions at  $300^\circ \times s^{-1}$  lasted approximately 80 s.

With the exception of study III, the highest value obtained during the initial five contractions was compared with the mean value of any three consecutive contractions during the entire test (i.e. 23-25, 48-50 etc). In study III the peak torque of one contraction was substituted by the mean of the three initial contractions as criterion for the peak torque of the experiments.

Muscle fatigue was expressed as the absolute as well as the relative torque decline from the initial peak torque and defined as "fatigue index". A high test-retest reliability for the fatigue test has earlier been demonstrated (Thorstensson 1976). A coefficient of variation of 3.2% was obtained based on day-to-day-measurements. In order to evaluate whether subjects in the present study did exert MPT in the fatigue test: initial peak values from a fatigue test were compared with MPT values as well as initial peak values obtained in a repeated fatigue test some days later. The  $\pm$  v corresponded to 3.7 and 3.9% (n = 22) respectively. In addition, 10 subjects were re-tested after 18 months. During the time elapsed physical activity was maintained at an almost constant level comparable to the pre-test level. The reproducibility of the fatigue test expressed as c v was 6.9% while the corresponding value found for MPT was 5.3%.

#### Maximal oxygen uptake (I, II, IV, VI)

Maximal oxygen uptake was determined using leg exercise on a Monark cycle ergometer by stepwise increasing the work load according to the levelling off criterion (Åstrand & Saltin 1961). Expired air was collected in Douglas bags and the volume measured in a spirometer. For  $O_2$  and  $CO_2$  analyses a mass spectrometer (Centronic 200 MGA, Centronic Works, Croydon, England) was used. Duplicate determinations revealed a c v of 1.2% (n = 20).

#### Cycling exercise (VI)

To make comparisons possible with previous muscle fatigue studies, short term maximal cycle exercise was applied. This took place without any warm up period. An exhaustive cycling test was performed on a Monark cycle ergometer with a work load corresponding to 120 (range 118-121) % of  $\dot{V}O_{2\max}$ . Inability to maintain a prescribed pedalling frequency (i.e. 70 rev  $\times$  min<sup>-1</sup>) was taken as criterion for exhaustion. The day-to-day variation in exercise tests of this kind (n = 10) was 3.1% (c v).

#### Downhill skiing (VII)

To study the effect of short term heavy muscle exercise in sport events, subjects performed submaximal and maximal skiing of approximately 1 min duration on a slope adjusted for competition.

The glycogen depletion pattern (Gollnick et al. 1972a) was studied during two consecutive days of skiing in order to evaluate muscle fibre recruitment pattern. The exercise consisted of ski-school activity (physical-education students) and competitive slalom and giant slalom training (elite skiers). Training sessions for both groups lasted approximately 5 h per day.



### EMG recordings (III-IV)

To evaluate changes in electromyographic activity during the muscle fatigue experiments EMG was registered from the vastus lateralis muscle. Surface electrodes (Hallige Stockholm) of 10 mm in diameter were placed over the belly of the muscle as close as possible to the site of the muscle biopsy insertion. Muscle biopsies (see later) were taken at least one week prior to EMG experiments. The interelectrode distance was 10 mm in the direction of the patella-crista iliaca.

Amplified EMG signals were either rectified and filtered by a single lag filter (120 ms time constant) and displayed on a UV Honeywell 2012 recorder along with the corresponding torque curve or amplified and immediately stored on magnetic tape (Philips Analog 7) with a recording speed of  $380 \text{ mm} \times \text{s}^{-1}$  for further analysis. Peak EMG was defined as the highest point on the rectified and filtered EMG curve while integrated EMG (IEMG) was taken as the area under the rectified and filtered EMG curve as measured by planimetry. Analysis was limited to the mid-range of knee extension.

Using a laboratory computer (HP 2116 C) and a data processing system (Komi & Lehtis 1973 Viitasalo & Komi 1975) IEMG and power spectral density function (PSDF) were obtained according to Bendat & Piersol (1971). In order to demonstrate changes in PSDF mean power frequency (MPF) was computed and the relative proportions were calculated for selected bandwidths (24-48 Hz, 56-88 Hz, 96-128 Hz and 136-400 Hz). For further description see Viitasalo & Komi (1977).

### Muscle biopsy sampling (I-VII)

Muscle tissue specimens (approximately 30-50 mg) were taken from vastus lateralis using the percutaneous needle biopsy technique (Bergstrom 1962). This part of the quadriceps muscle has been found to be heavily activated when performing knee extensions during isokinetic conditions (Marrifield & Dostal 1978), maximal cycling exercise (Bigland-Ritchie & Woods 1974, Henriksen & Bonde-Petersen 1974) and downhill skiing (Eriksson et al. 1976).

Biopsies for histochemistry were mounted in embedding media (Cryofix<sup>TM</sup> Damon, Needham Hts. Mass.) and frozen in isopentane cooled with liquid nitrogen. For biochemistry muscle biopsies were frozen directly in liquid nitrogen. Samples were stored at  $-80^{\circ}\text{C}$  until analysed. Specimens for glycogen determinations, histochemical as well as biochemical were obtained within 5 min before and after exercise. Muscle biopsies for lactate assays were obtained within 3-5 s after exercise with the subjects in a sitting position while muscle biopsies after skiing were taken within 10-15 s in a supine position.

### Histochemistry (I-VII)

Serial transverse sections (10  $\mu\text{m}$ ) were cut in a microtome at  $-25^{\circ}\text{C}$ . Stainings were undertaken for ADPase activity (Gocori 1941, Padykula & Herman 1955) after preincubation at pH 10.3, 4.6 and 4.3 respectively (Engel 1962, Brooke & Kaiser 1970) as well as for NADH-tetrazolium reductase (Novikoff et al. 1961). For classification fibres were

identified as fast twitch (FT) and slow twitch (ST) fibres (Engel 1962) and for further subgroup classification of FT fibres (Brooke & Kaiser 1970) into F1a and F1b according to the terminology proposed by Saltin et al (1977). The percentage of each fibre type was calculated from sections containing at least 200 fibres but mostly comprising 400-500 fibres. Muscle fibre area was determined on NADH-tetrazolium reductase stained fibres using a cutting and weighing procedure (Thorstenenson 1976). At least 10 fibres of each fibre type subjectively rated as representative for the entire transverse sections were selected for analysis. The relative muscle area occupied by FT muscle fibres was calculated according to the formula:  $(100 \times \text{FT/ST area} \times \% \text{ FT}) \times (\text{FT/ST area} \times \% \text{ FT} + \% \text{ ST})^{-1}$ . Error of methods (c v) estimated from analysis of duplicate biopsies ranged 6-11% for area and distribution variables (Table 2).

Table 2 Methodological errors (c v) of histochemical and biochemical variables. Samples used for lactate assay ranged 10-35 in concentration (moles  $\times \text{kg}^{-1}$  wet muscle). Analyses were conducted on pools of fibres ranging 10-30  $\mu\text{g}$  in dry weight. Mean values were calculated from 2-3 pools of fibres and therefore each value represents a dry weight corresponding to approximately 40-80  $\mu\text{g}$ .

<u>HISTOCHEMISTRY</u>	<u>c v %</u>	<u>n</u> <sup>2)</sup>
Muscle fibre type distribution <sup>1)</sup>	6.2	28
Muscle fibre area <sup>1)</sup>	10.5	30
Muscle fibre area ratio <sup>1)</sup>	7.5	26
<u>LACTATE</u>		
<u>ANALYSES UNDERTAKEN SIMULTANEOUSLY</u>		
Pool to pool: unidentified fibres	6.8	35
— FT fibres	8.7	35
— ST fibres	7.2	35
— divided fibres	3.6	12
Mean to mean: unidentified fibres	4.4	32
— FT fibres	4.4	12
— ST fibres	4.4	12
<u>ANALYSES UNDERTAKEN ON TWO SUCCESSIVE DATES</u>		
Pool to pool: divided fibres	6.7	14
Mean to mean, —	2.2	6
<u>ANALYSES UNDERTAKEN ON DIFFERENT OCCASIONS</u>		
Mean to mean	5.6	12

1) Duplicate biopsies

2) Duplicate determinations

To demonstrate selective glycogen depletion pattern cross-sections (16  $\mu$ m) were PAS (periodic acid-Schiff) stained for glycogen (Pearse 1961). Individual fibres were subjectively rated as filled, moderately emptied, and exhausted using a system similar to that proposed by Gollnick et al. (1972a).

#### Biochemistry (I II VI VII)

Metabolites were determined in mixed muscle samples as well as in separated, dissected out fibres. After weighing at  $-25^{\circ}\text{C}$  on a Cahn electrobalance, muscle biopsies (approximately 10-50 mg) were analysed for their glycogen and lactate contents by means of fluorometer technique according to Lowry & Passonneau (1972) as modified by Karlsson (1971) using a Farrand ratio fluorometer model 24 (Farrand Optical Co. New York).

Analyses on separate muscle fibres were performed in the following way: after lyophilizing muscle samples (approximately 2-12 mg) were placed under a microscope and 100-200 fragments of muscle fibres were dissected out from each sample at a constant room temperature and humidity ( $20^{\circ}\text{C}$  and 30% respectively). Thereafter a part of each fibre was cut off and stained for fibre type identification (Esaén et al. 1975a). For lactate analysis 5-13 FT and ST fibre fragments respectively were pooled and weighed on a Cahn electrobalance or Quartz fibre ultramicro balance (Bodder "E" Microtech Services, Berkeley). Weight of the samples corresponded to 10-30  $\mu$ g. Fibres were then put into test tubes before the reagent solution was added. The lactate concentration was determined according to Karlsson (1971). Each value was calculated as a mean value for the lactate concentration in 2-7 pools. Comparing values obtained from conventional analysis on whole wet (Karlsson 1971) or freeze-dried (Esaén 1978) samples with the present method, no systematic differences were noted in terms of lactate concentration, although wet sample values tended to be lower, thus confirming Karlsson (1971). Other methodological errors for lactate analysis estimated from duplicate determinations are listed in Table 2. All values were converted into wet weight values assuming a water content of 77% for muscle biopsy samples (Karlsson 1971). This value for muscle biopsy water content was confirmed and no differences were obtained irrespective of whether biopsies ( $n = 25$ ) were taken at rest or after short term (60 s) heavy exercise. However, small intra- and interindividual variations in water content were present, corresponding to 75-78%.

In order to express lactate concentration for the entire muscle investigated, the following formula was applied: Lactate concentration in FT fibres  $\times$  % FT area, lactate concentration in ST fibres  $\times$  % ST area. Lactate dehydrogenase (LDH) activity and muscle specific LDH (M-LDH) activity for the entire muscle were calculated in a manner corresponding to lactate.

For muscle tissue enzyme studies, additional dissected pools of FT and ST fibres each consisting of approximately 100 fibre fragments were homogenized in 100  $\mu$ l of 0.5 M KCl. Total lactate dehydrogenase (LDH) (E.C. 1.1.1.27) activity in the forward reaction (pyruvate  $\rightarrow$  lactate,  $\text{LDH}_{\text{tot}}$ ) was determined fluorometrically.

(Lowry & Passonneau 1972) in the homogenate diluted 1:4 with 0.1 M Tris-HCl buffer pH 7.5. Discelectrophoresis for separating the LDH isoenzymes was performed according to Dietz & Lührsen (1967) on 25  $\mu$ l of the homogenate. The relative contribution of muscle specific LDH monomers (% M-LDH) was determined using a densitometric scanning technique on the separated and stained LDH isoenzyme bands in the gels (Sjödén 1976). The activity corresponding to the muscle specific LDH (M-LDH) was then calculated (% M-LDH  $\times$  LDH<sub>tot</sub>). The error of method ( $\pm$ ) estimated from duplicate biopsies was 3.1% (n = 10) (Sjödén personal communication).

### Blood analysis

Blood samples were obtained from the finger tip and analysed for lactate concentration according to the Barker and Summerson method as modified by Ström (1949).

## RESULTS AND COMMENTS

### Muscle fibre type distribution in vastus lateralis of man (I-VII)

Muscle fibre type distribution in the vastus lateralis muscle in 64 healthy young men averaged 49 (range 10-79) % FT fibres. The relative area occupied by FT fibres averaged 52 (range 10-77) %. When subjects involved in competitive physical training were excluded, the values corresponded to 53 (range 29-79) % FT and 57 (range 28-77) % FT area respectively. Individual FT/ST muscle fibre area averaged in the whole material 1.20 (range 0.70-1.85) and 1.24 (range 0.88-1.85) when trained subjects were excluded (Table 3).

Table 3 Mean (range) values for muscle fibre type distribution and area in the sample studied including (n = 64) and excluding 14 individuals involved in competitive physical sports

	Fibre type distribution % FT	FT fibre area 100 $\mu$ m <sup>2</sup>	ST fibre area 100 $\mu$ m <sup>2</sup>	FT area %	FT/ST area
n = 64	49 (10-79)	64.5 (35.9-101.7)	54.4 (29.4-88.9)	52 (10-77)	1.20 (0.70-1.85)
n = 50	53 (29-79)	66.8 (36.9-101.7)	55.5 (29.4-88.9)	57 (28-77)	1.24 (0.88-1.85)

Subgroup classification of FT muscle fibres was undertaken on samples from 23 subjects. The mean distribution of FTa and FTb fibres was 30 (range 13-49) % and 19 (range 0-37) % respectively.

Comments: The subjects were recruited among physical education students or individuals with approximately an equivalent physical activity level. Fourteen athletes involved in competitive physical training were also included.

The results concerning fibre type distribution and fibre area in m. vastus lateralis of men revealed a pattern in agreement with previous studies (Edström & Ekblom 1972; Gollnick et al. 1972b). Although there is a wide range in the distribution, muscles of physically active though not competitive men seem to be composed of a smaller proportion of FT fibres as compared to muscles of individuals in the sedentary state as also reported by e.g. Gollnick et al. (1972b) and Larsson (1978). Furthermore, it was noted that individuals successful in endurance events displayed a more homogeneous fibre type distribution, characterized by a high percentage of FT fibres and a low occurrence of FTb fibres, thus confirming other authors (Jensen 1975; Mygaard-Jensen 1976; Jansson & Kaijser 1977; Jansson et al. 1978).

#### Muscle strength and its relation to muscle fibre type distribution (I-V)

Peak torque, attained at a relatively high speed of movement ( $180^\circ \times s^{-1}$ ), either expressed per kg body weight or in absolute terms, was positively correlated to the percentage of FT fibres as well as to the relative proportion of FT fibres ( $r = 0.48-0.65$ ,  $p < 0.001$ ,  $n = 50$ ). In addition, peak torque was related to the absolute FT fibre area ( $r = 0.30-0.33$ ,  $p < 0.05$ ).

In a subgroup ( $n = 18$ ) peak torque at a higher angular velocity ( $300^\circ \times s^{-1}$ ) was studied. This speed is approximately 50% of the maximum angular velocity that could be obtained during knee extension (Thorstensson 1976). Peak torque averaged 131 Nm or  $1.8 \text{ Nm} \times \text{kg}^{-1} \text{ b.w.}$  as compared to 151 Nm or  $2.1 \text{ Nm} \times \text{kg}^{-1} \text{ b.w.}$  at  $180^\circ \times s^{-1}$ . The observed relation between the proportion of FT fibres and peak torque developed was not further strengthened by increasing angular velocity. Approximately the same correlation coefficients were found when relating peak torque to percentage of FT fibres as well as to the relative area occupied by FT fibres. No relationship could be demonstrated between peak torque and the relative occurrence of one or the other type of FT fibre subgroups.

Comments: Peak torque developed at movements of relatively high angular velocities ( $180-300^\circ \times s^{-1}$ ) was found to be related to the percentage of FT fibres, thus confirming Thorstensson & Karlsson (1976). Since peak torque also was related to the absolute FT fibre area, but not to mean fibre area, there are reasons to believe that the quality of the activated muscle, i.e. the proportion of FT fibres, is of primary importance for force development at higher angular velocities. This suggestion is in accordance with recent observations by Coyle et al. (1979). Consequently, the quantity, i.e. the muscle mass as such, is suggested to be of secondary importance in terms of

force development at high angular velocities. Even though it has been possible to correlate mean fibre area with the cross-section of the muscle by means of dantor tomography (Nygaard et al. 1978a, Båggren et al. 1978) such an interpretation must however be taken with caution.

#### Muscle power and fatigue and the relation to fibre type distribution (II-VI)

Decrease in peak torque was found to occur within 10 maximal muscle contractions ( $p < 0.001$ ,  $n = 50$ ). Muscle fatigue either expressed as the relative or absolute decline in peak torque after 50 contractions was positively correlated to the muscle fibre type distribution expressed as % FT ( $r = 0.43-0.61$ ,  $p < 0.01-0.001$ ) or % FT area ( $r = 0.40-0.58$ ,  $p < 0.01-0.001$ ) ( $n = 50$ ). The relationship observed between fibre type distribution and relative decline in peak torque confirms the findings by Thorstensson (1976). In a subsample muscle fatigue was found to be related also to the occurrence of Fth muscle fibres (Fig. 1). Approximately the same correlation coefficients were present when relating the decline in peak torque either expressing the fibre type population as above ( $r = 0.53-0.57$ ,  $p < 0.01$ ) or to the percentage of Fth fibres ( $r = 0.53-0.57$ ,  $p < 0.01$ ).

Changes in peak torque were as expected accompanied by corresponding changes in power and work. Hence decline in power and work was correlated to the percentage of FT fibres ( $r = 0.64-0.75$ ,  $p < 0.05-0.01$ ,  $n = 12$ ). The amount of work produced during the first 15 contractions (20 s) was approximately 20% greater ( $p < 0.05$ ) in a group of subjects ( $n = 5$ ) with muscles made up by a high proportion of FT fibres (mean  $\pm$  SE 62-4%) as compared to individuals ( $n = 5$ ) with a low proportion of FT fibres (37-3%). With additional contractions this difference became smaller and smaller. After 100 contractions the cumulated amount of work was approximately equal comparing the two groups (Fig. 2).

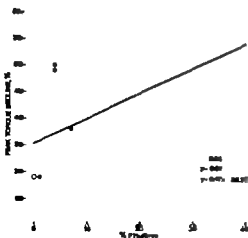


Fig. 1 The relationship between the percentage % FT muscle fibre in the vastus 1 et 2 muscles and the decline in peak torque with 50 maximal knee extensions at an angular velocity of  $180^\circ \text{ s}^{-1}$  ( $n = 23$ ). Individual with less than 33% FT fibres are denoted with open (O) dots.

IDH and its significance for lactate formation (I II VI unpublished results)

Correlation coefficients between  $IDH_{tot}$  as well as M-IDH activity and individual muscle fibre type distribution (% FT) were 0.96 ( $p < 0.001$ ) and 0.98 ( $p < 0.001$ ) respectively. In addition the relative contribution of M-IDH in FT and ST fibres respectively was related to the percentage of FT fibres in the muscles investigated ( $r = 0.74$ ,  $p < 0.01$  and  $r = 0.89$ ,  $p < 0.001$ ) i.e. the proportion of FT fibres in the muscle reflected the quality of the ST fibre in respect to its IDH activity.

To study further the significance of IDH enzyme activity on lactate formation capacity 10 subjects performed 25 repeated muscle contractions according to Thorstensson (1976). Mean values were 18.5 (range 3.5-31.1)  $\mu\text{moles} \times \text{kg}^{-1}$  wet weight (lactate)  $\pm$  9.0 (range 0.38-1.65) ( $IDH_{tot}$ ) and 0.60 (range  $\pm$  0.18-1.54) (M-IDH)  $\mu\text{moles} \times \text{kg}^{-1} \times \text{min}^{-1} \times 10^4$  wet weight. The individual differences in muscle lactate accumulation after exercise were found to be related to individual variations in  $IDH_{tot}$  ( $r = 0.66$ ,  $p < 0.05$ ) and M-IDH ( $r = 0.70$ ,  $p < 0.05$ ) activity. This relationship was also present in 7 endurance trained individuals. At exhaustion following a short term cycling exercise the individual variation in lactate concentration (mean and range were 20.8 and 13.9-30.5  $\mu\text{moles} \times \text{kg}^{-1}$  wet weight) was found to be positively correlated to the percentage M-IDH ( $r = 0.73$ ,  $p < 0.05$ ) (Table 4).

Comments: The data confirm earlier findings by Karlsson et al. (1974) and Sjödin (1976) that muscle fibre type distribution will determine the metabolic quality of the muscle in terms of its potentials for lactate formation. In addition to this an interaction appears to be present between the two muscle fibre types. Thus in ST fibres of muscles rich in FT fibres the IDH activity as well as IDH isoenzyme pattern approached the properties of FT fibres more than in a muscle rich in ST fibres. Whether or not this is an effect of adaptive mechanisms due to the environment remains speculative.

Table 4 The interrelationship between some physiological, histochemical, biochemical and functional parameters obtained in 5 individuals performing short term high intensity exercise

Subject	$\dot{V}O_2 \text{ max}$ $\text{ml} \times \text{kg}^{-1} \times \text{min}^{-1}$	FT %	M-IDH %	Lactate concentration $\mu\text{moles} \times \text{kg}^{-1}$ wet muscle			Performance time s
				FT	ST	whole muscle	
I	70 $\pm$	29	39	14.4	13 $\pm$	13.8	99
II	85.9	29	45	26.2	15.5	18.8	101
III	66 $\pm$	40	39	26.7	21.4	23.8	119
IV	60.6	42	56	26.0	23.5	24.6	124
V	61.0	62	74	31.8	26.5	30.5	126

Taken together the data from the isokinetic one leg and cycling experiments seem to indicate that the activity and quality of the LDH-enzyme are critical for lactate formation and performance capacity. Previously it has been demonstrated that physiological concentrations of lactate *in vitro* will decrease LDH activity (Karlsson et al 1974). Furthermore it has been shown that phosphofructokinase (PFK) activity considered to be the rate limiting enzyme for glycolysis is inhibited at low pH (Denforth 1965). A decrease in pH has been demonstrated concomitant to lactate formation in voluntary contracting human muscle (Sahlin 1978).

#### Myoelectric activity during fatiguing conditions (III-IV)

Although a pronounced decrease in peak torque was demonstrated after 50 and 100 contractions corresponding to 50-75% peak EMG and IEMG decreased only slightly (n.s.) in the whole material studied ( $n = 23$ ). In a subsample of individuals ( $n = 6$ ) with muscles with a high proportion of FT fibres IEMG decreased significantly ( $p < 0.01$ ). As a consequence EMG activity for the whole material either expressed as peak EMG per unit peak torque or IEMG per unit work increased progressively to level off around the 75th contraction. The change (increase) in these ratios with 50 contractions was positively correlated to individual muscle fibre type distribution expressed as per cent FT fibres ( $r = 0.84$   $p < 0.001$  and  $r = 0.78$   $p < 0.01$ ). Consequently the increase in the IEMG/torque ratio was positively related to the relative decrease in peak torque ( $r = 0.88$   $p < 0.001$ ) following 100 contractions.

Different qualitative EMG changes were observed during repeated muscle contractions thus a 25% decrease ( $p < 0.001$ ) in power spectral density function (PSDF) of the EMG expressed as mean power frequency (MPF) was present in a group of individuals ( $n = 6$ ) with muscles rich in FT fibres (mean  $\pm$  SE relative FT fibre area  $67 \pm 3\%$ ). In a group of subjects ( $n = 5$ ) with muscles not as rich in FT fibres (43-1%) a smaller nonsignificant decrease of MPF was present. Intergroup deviations were noted already after 25-30 contractions ( $p < 0.01$ ). When these two groups were combined individual relative change in MPF after 25 contractions was positively related to peak torque decline ( $r = 0.74$   $p < 0.01$ ) as well as to muscle fibre type distribution expressed as per cent FT fibres ( $r = 0.60$   $p < 0.05$ ).

Comparing the frequency spectrum changes between the two groups certain differences were observed. Increase in the relative content of the lower bandwidth (24-48 Hz) as well as decrease in the higher bandwidth (136-400 Hz) were significantly greater ( $p < 0.01$  and  $p < 0.05$  respectively) in subjects characterised by muscles rich in FT fibres.

In addition to qualitative changes in EMG activity events linked to the neuromotoric control were found to be altered with a concomitant reduction in muscle force. Thus individual differences related to muscle fibre type distribution were noted when studying the time lag between the first action potential of the EMG and the corresponding torque recording at a pre-set angular velocity ( $180^\circ \times s^{-1}$ ) ( $n = 12$ ). Values demonstrated initially and after 50 and 100 contractions were (mean  $\pm$  SE)



95 $\pm$ 3 115 $\pm$ 5 and 121 $\pm$ 5 ms respectively. The time lag was initially negatively related to the percentage of FT fibres ( $r = -0.68$   $p < 0.05$ ) and the percentage time lag increase after 100 contractions was positively related to % FT ( $r = 0.73$   $p < 0.01$ ).

**Comments:** The fatigue associated shift in EMG frequency spectrum towards low frequency components is in accordance with Kogi & Hakamada (1962). A significant drop in EMG activity as well as a more pronounced shift in the frequency spectrum of EMG with fatigue was noted in muscles with a high percentage of FT fibres as compared to muscles with a high percentage of ST fibres. Although information regarding lactate concentration was not available from these experiments, it can be assumed that after 25 contractions higher values were present in those subjects who demonstrated the most pronounced shifts in PSDF. This reasoning seems to hold true since data obtained on individuals also taking part in experiments where lactate concentration was determined revealed an inter-relationship between force decline, lactate accumulation (and LDE activity) and PSDF shift (Fig. 5). Whether changes in time lag between onset of EMG and torque development at a pre-set angular velocity depend on biochemical processes taking place as a result of repeated muscle contractions cannot be established here. Nevertheless, the present data clearly indicate that the muscle rich in FT fibres initially has the shortest time lag but suffers more during repeated contractions as compared to the muscle rich in ST fibres.

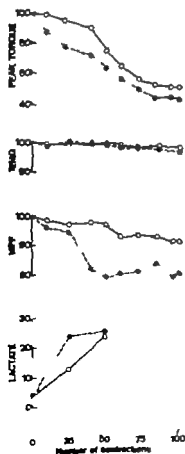


Fig. 5. Changes (expressed as % of initial value) for two individuals in peak torque integrated EMG (IEMG) and mean power frequency (MPF) during repetition of 100 maximal knee extensions. Muscle biopsy for lactate concentration ( $\text{mmol} \cdot \text{s}^{-1} \cdot \text{kg}^{-1}$  wet muscle) during contractions was obtained in separate experiments. The two subjects differed in respect to muscle fibre type distribution; subject GC (34% FT fibres) and subject CL (34% ST fibres).

### Lactate formation during downhill skiing (VII)

In order to study the effects of acute physical activity in sports demanding great muscular power downhill skiing was used as an experimental model. Low muscle glycogen values in the vastus lateralis muscle were noted after a day of skiing and in two samples studied levels below 15 moles glucose units  $\times \text{kg}^{-1}$  wet weight were found. Skilled and unskilled skiers demonstrated different selective glycogen depletion patterns. In both groups a great loss of glycogen from the FT fibres was demonstrated. However, in the skilled skiers a pronounced depletion of ST fibres was observed as well. Based on these findings it was suggested that downhill skiing to a high degree recruited the FT fibre population irrespective of training status. This was supported by the fact that it was not possible to relate muscle lactate accumulation to level of proficiency. However, lactate concentration in the exercised muscle was positively correlated to the relative distribution of FT muscle fibres after maximal skiing (Fig. 3). No such relationship was demonstrated following submaximal skiing conditions resulting in significantly lower lactate values. Furthermore, the FT/ST lactate ratio reached higher values during maximal skiing in individuals rich in FT fibres as compared to their opposites ( $r = 0.84$ ,  $p < 0.05$ ).

Comments: Low muscle glycogen levels were demonstrated after skiing and such values are only reported after prolonged intense running, cycling or cross-country skiing. Thus it can be concluded that downhill skiing, competitive as well as recreational, is performed with an enhanced utilization of glycogen especially in FT fibres and that the site of the quadriceps muscle chosen is representative to study muscle metabolism during downhill skiing.

Downhill skiing is evidently a physical activity which is dependent on recruitment of FT fibres irrespective of training status and is therefore in some respects comparable to the different laboratory tests applied in the present study. Accordingly it suggests that the FT fibres were more active in formation of lactate. It can be suggested that downhill skiing, especially in less skilled skiers, will be characterised by a high degree of isometric muscle exercise. This assumption is supported by the fact that a high FT/ST lactate ratio was observed in subjects rich in FT fibres which is in accordance with findings obtained during standardised isometric exercise at different tension levels (Neech & Karlsson 1977).

The present data indicated that skilled skiers recruited relatively more of their ST fibre population than unskilled. Nygaard et al. (1978b) observed pronounced glycogen depletion of FT fibres in one inexperienced skier while none or minor loss of glycogen from these types of fibres was observed in more advanced skiers. This provides further evidence of a contrasting recruitment pattern in skilled and unskilled individuals.

## GENERAL DISCUSSION

*Metabolic profiles of muscle fibre types in man*

Studies on separated human skeletal muscle fibre types disclose a consistent pattern regarding their metabolic profiles. Thus in terms of metabolic potentials the ST fibre is suited for use in prolonged activities with reliance upon oxidative metabolism, indicated by higher activity of enzymes involved in the citric acid cycle e.g. succinate dehydrogenase (Esaén et al. 1975a, Henriksson & Reitman 1976) as well as a LDH isoenzyme pattern favouring lactate oxidation (Sjöödin 1976). Furthermore observations on habitually active men have indicated a greater blood flow in muscles rich in ST fibres as compared to muscles rich in FT fibres (Juhlin-Dannfelt et al. 1979). Thus a relationship between leg vascular resistance and muscle fibre type distribution was demonstrated which fits well with the greater capillary density of muscles rich in ST fibres (Andersen 1975, Andersen & Henriksson 1977). Moreover a relationship between the mitochondrial content and number of capillaries surrounding each fibre has been demonstrated (Brodeur et al. 1977). The FT fibre on the other hand, in addition to superiority in terms of contractile characteristics (Thorstensson et al. 1977) display higher activity of enzymes thought to be rate limiting for glycolysis i.e. phosphorylase (Piehl & Karlsson 1977), phosphofructokinase (Esaén et al. 1975a, Henriksson & Reitman 1976) and lactate dehydrogenase (Sjöödin 1976, Thorstensson et al. 1977). Even in terms of stored metabolites differences are present between the two main fibre types. Slightly higher CP concentrations were noted in FT fibres while no differences in ATP concentration of fibre types were noted in the resting muscle (Esaén 1978). Triglyceride content is significantly higher in ST fibres (Esaén et al. 1975a) whereas glycogen content seem to be approximately equal in different fibre types (Esaén et al. 1975a, Piehl & Karlsson 1977).

In addition the subgroups of FT fibres are known to differ in terms of enzymatic and vascular profile. In most respects the FTA fibre seems to represent an intermediate type in comparison to the ST and FTb fibre (cf. Saltin et al. 1977).

Muscle fibre type distribution as determined by staining for myofibrillar ATPase in the muscle reflects the acto-myosin ATPase activity which in turn is assumed to be correlated with intrinsic speed of muscle shortening (Bärry 1967, Bärry & Close 1971) in the investigated muscle. Considering this the present results clearly demonstrate that individual fibre type distribution to a certain extent will be important for anaerobic performance capacity as indicated by repeated muscle contractions of high intensity.

*Muscle fibre recruitment*

Established methods are available for evaluation of the muscle fibre recruitment pattern in vivo. The degree of glycogen depletion in different fibre types by histochemical means is one method to evaluate whether a certain type of fibre has been metabolically active during exercise (Rugdalberg & Edström 1968).

Gollnick and co-workers (Gollnick et al. 1973a, b 1974a, b) found with this approach that ST fibres seem to be recruited at muscle contractions calling for less than 20% of maximal voluntary isometric contractions. At tensions exceeding 20% FT muscle fibres are recruited in addition. In dynamic (cycling) exercise with an energy need below maximal oxygen uptake ( $\dot{V}O_2 \text{ max}$ ) ST muscle fibres are the first to loose glycogen. At supramaximal load corresponding to 120% of  $\dot{V}O_2 \text{ max}$  muscle glycogen depletion pattern indicates an initial loss from FT fibres although there are reasons to believe that a major portion of the ST fibre population is recruited simultaneously. The rate of pedalling ( $30-120 \text{ rev} \times \text{min}^{-1}$ ) does not influence the glycogen depletion pattern. Moreover at intensities close to or above  $\dot{V}O_2 \text{ max}$  both FT fibre types are recruited (Andersen & Sjgaard 1976). However it is not possible to establish with this approach whether certain muscle fibre types are continuously involved during an entire pedal thrust or engaged only in short bursts. Using the same experimental isokinetic exercise protocol as in the present study contraction velocity was found to influence muscle fibre recruitment pattern suggesting a greater involvement of FT fibres at faster velocities (Lammi et al. 1979).

Intramuscular substrates other than glycogen may have been utilized as indicated by Esseen (1978) analysing single muscle fibres for their metabolic contents. Furthermore a higher energy consumption per unit isometric tension developed has been demonstrated for FT as compared to ST fibres of hamster (Goldspink et al. 1970) which may obscure the recruitment pattern as indicated by glycogen staining intensities. In line with this is the finding that the rate of heat production in human skeletal muscles is 6 times higher in FT as compared to ST fibres (Boasted & Erland 1978). The rate of glycogen breakdown is also found to partly be dependent on individual muscle fibre type distribution (Juhlin-Dannfelt et al. 1977) and consequently care must be taken when evaluating for fibre recruitment pattern using the glycogen depletion method.

In order to evaluate the orderly recruitment pattern of single motor units electromyography using fine needle electrodes (Benners 1974) and bipolar surface electrodes (Gydikov & Rosarov 1974) has been studied. Information on muscle fibre recruitment pattern in man has been obtained in isometric (cf Gydikov & Rosarov 1974, Grisby & Benners 1977) but also in isotonic (Grisby & Benners 1977) leg exercise. The results confirm the size principle theory based on experiments on decerebrated cat experiments (Henneman et al. 1965). In addition, an initial selective recruitment of FT motor units was demonstrated during ballistic movements irrespective of muscle tension level (cf Grisby & Benners 1977). These results indicate that an exclusive pool of FT motor units always will fire at onset of a muscle contraction when muscle tension is rapidly increased and/or forceful. FT fibres were also observed to be activated upon sudden and vigorous contractions in partially denervated human muscles (Marras & Engel 1972).

## Muscle exercise and metabolism

The present findings indicate that it is possible to objectively detect a decrease in the individual's ability to produce maximum muscle force within 10 muscle contractions. For such a short period of intense exercise, no experimental data are available showing complete depletion of the immediate energy stores. A certain reduction of muscle ATP and CP depots are, however, present after 10-15 s of heavy exercise (Bultman et al. 1967, Bergström et al. 1971, Saltin et al. 1971).

The question may be raised whether or not a complete lack of ATP in the myofibrillar regions is possible. Based on frog and rat experiments, impairment of contractile function due to inadequate availability of myofibrillar ATP has been suggested by e.g. Pitts & Bollosay (1976, 1977). Murphy (1966), stimulating frog sartorius muscles, demonstrated a close relationship between tension output and the ATP content of a metabolically inhibited muscle. However, he concluded that it is unlikely that any ATP dependent limitations will be present in the mechanical response of muscles with intact ATP restitution mechanisms.

Spende & Schottelius (1970) stimulated isolated mouse muscle to fatigue and observed a linear relationship between CP concentration in the muscle and tension developed in an isometric tetanus. They proposed that CP depletion can be the underlying cause of fatigue. Whether the contractile machinery was directly affected or not could not be established from their results.

Information concerning the glycogen depletion pattern during brief exercise in the order of 10-25 contractions are not available but most probably muscle contractions, irrespective of contraction velocity and tension output, must be repeated for longer periods of time in order to exhaust certain fibres (Andersen & Sjogaard 1976, Secher & Nygaard-Jensen 1976, Essén 1978).

It is open to speculation whether exhaustion following supramaximal cycling exercise is partly attributed to substrate depletion of highly recruited FT fibres. By repeating exhaustive supramaximal one minute bouts, approximately 50% of the FT fibre population was found to be exhausted of their glycogen content already after 5 bouts (Thomson et al. 1979). Since this fibre type to a high extent seems to contribute to the force applied at each pedal thrust (see above), selective substrate depletion cannot be excluded as being a limiting factor in intense cycle exercises in the order of 1-2 min duration.

## Force generation and motor unit activity

In order to study the origin of fatigue, changes in EMG activity and muscle force have been followed during various type of activity resulting in impairment of muscle function. During sustained constant submaximal isometric contraction, increased EMG activity occurs (Horton 1954, Edwards & Lippold 1956, Lippold et al. 1960, Kuroda et al. 1970, Viitasalo & Komi 1977). This has been assumed to be due to recruitment of additional motor units and/or increased firing frequency to compensate for failure in contractility of already recruited fibres (Lippold et al. 1960). In experiments

where the fatigue mechanism was studied in sustained maximal isometric contraction in man a fall in EMG activity concomitant to a loss in muscle force was observed (Stephens & Taylor 1972). The unchanged electrical activity to tension (E/T) ratio was taken as indicative of fatigue at the neuromuscular junction. Ochs et al. (1977) demonstrated also a drop in electrical activity when studying EMG and force relationship during rapid repetitive maximal isometric plantar flexion. In comparison with Stephens & Taylor (1972) muscle tension was however found to decrease at a higher rate as compared to the EMG activity which implies an increased E/T ratio. Hence muscle fatigue was indicated to be located peripheral to the neuromuscular junction.

Dissimilarities regarding changes in E/T ratio during development of muscle fatigue may be influenced by factors such as type of contractions investigated (e.g. submaximal vs. maximal isometric contraction, isometric vs. dynamic contraction, slow vs. fast dynamic contraction). It has also been suggested that migration of activity may occur within a muscle as well as from one muscle to another (Lippold 1955, Rossi & Viitasalo 1976).

Studying human single motor units a decrease in firing frequency (Marras et al. 1971) has also been noted concomitantly with a fall in muscle tension (Gydlkov & Rosenov 1974) indicating that EMG might fall without any neuromuscular block. Contradicting findings regarding E/T ratio and conclusions in the literature might be due to differences in type of motor units dominating in the investigated muscle. By comparing the EMG activity of the human gastrocnemii and soleus known to differ in terms of histochemical properties (Gollnick et al. 1974c, Edgerton et al. 1975) Ochs et al. (1977) observed a more pronounced increase in E/T ratio of the gastrocnemii muscle during repeated plantar flexions. EMG studies of human dorsal interosseous muscles have shown that FT motor units fatigue more easily than ST units. In addition a broader range in fatigability was observed for FT motor units as compared to ST motor units (Stephens & Usherwood 1975). Decrease in the EMG activity during maximal effort is suggested to be due to inhibition of some motor units and/or changes in the form of motor unit potential e.g. amplitude, duration, frequency spectrum. Support for the latter possibility was demonstrated in experiments leading to conditions referred to as ischaemic (Mortimer et al. 1970). In agreement with human experiments (Stålberg 1966) a decrease in the conduction velocity of stimulated cat muscle fibres was observed which was suggested to account for observed changes in the EMG frequency spectrum. The decrease in conduction velocity was more pronounced in the gastrocnemii as compared to the soleus muscle. The authors suggested lactate as responsible for these changes although no data were obtained on lactate concentration.

## Lactate accumulation and muscle fatigue

One of the basic findings in the present study was the higher rate of lactate formation observed in FT as compared to ST fibres. The results agree with the data obtained in animal experiments (Ogata 1960; Beatty et al. 1963) and previously suggested by Fletcher (1914). A lactate "flow" from FT to ST fibres may have occurred as discussed by Esbén et al. (1975b) mediated by membrane bound M-LDH as suggested by Björlin (1976) and thus partly explaining the lactate content in ST fibres.

Concomitant with a reduction in ATP and CP stores there is an increased lactate formation (Bultman et al. 1967; Karlsson 1971). Lactate accumulation depends on lactate production and release. As demonstrated, lactate release in man is found to be maximal already at muscle lactate concentrations corresponding to approximately  $5 \text{ mmol} \times \text{kg}^{-1}$  wet muscle (Jorfeldt et al. 1978). The authors did not indicate whether the assumed translocation hindrances for lactate were related to individual muscle fibre type distribution. Following submaximal cycling exercise greater muscle/blood lactate gradients were observed in individuals with muscles rich in FT fibres as compared to what was found in individuals with muscles rich in ST fibres (Graham et al. 1978). Accordingly the present results suggest that individuals with muscles rich in high oxidative (ST and/or FTA) fibres have a greater release, most probably due to a more developed capillary network (Andersen 1975; Andersen & Henriksen 1977). This suggestion is in agreement with the findings by Folkow & Balicka (1968) comparing red and "white" muscles of cat with respect to blood supply, capillary surface area and oxygen uptake at rest and during exercise.

A causal relationship between muscle fatigue and lactate accumulation has been suggested (Amussen et al. 1948; Karlsson 1971; Klausen et al. 1972). Support for this view has been found in human experiments where performance capacity of longer duration (3-5 min) was found to be negatively affected by elevated blood and muscle lactate levels induced by previous exercise of other muscles (Karlsson et al. 1975). Accordingly the same lactate concentrations were demonstrated during maximal exercise at various ambient pressures concomitant to increased performance time with increased ambient pressure (Linnarsson et al. 1974).

In muscle biopsy specimens a close relationship between pH and lactate concentration immediately at termination of exercise has been demonstrated (Sahlin 1978) and the same muscle vein pH was demonstrated on exhaustion during maximal exercise at varying ambient pressures (Kaijser 1970) indicating that increased acidity in the muscle may determine the point of exhaustion. The mechanism for lactate fatigue is however not clear. A mechanism for high  $\text{H}^+$  concentrations to cause impaired muscle function has been demonstrated by Fuchs et al. (1970) and Maksumura & Schwarz (1972). They showed that an increase in the  $\text{H}^+$  concentration decreases the number of calcium ions bound to troponin during the excitation-contraction coupling. This would reduce the number of active actin-myosin interactions thus decreasing contractile force. Experiments on skinned frog muscle fibres have supported this hypothesis (Robertson & Kerrick 1979). Thus intracellular acidosis depressed mechanical function at the

actomyosin interaction level. In addition a lowered pH was observed to require a higher  $\text{Ca}^{2+}$  concentration to result in a given level of activation. Eberstein & Sadow (1963) have published evidence indicating that the functional site of fatigue is in the link that couples excitation to contraction either reducing the intensity of the excitation-contraction link or decreasing the sensitivity to activation. Changes in flux of calcium were suggested to alter the operation of excitation-contraction link.

Recently Pitts & Holloway (1976-1978) were able to demonstrate a linear relationship between twitch and tetanic tension and lactate concentration of stimulated frog sartorius muscle. During 30 s of recovery however contractile twitch tension and ATP concentration increased rapidly while no significant reduction in lactate concentration occurred during this period. In experiments where cat gastrocnemii and soleus muscles were stimulated to contract a discrepancy in fatigue pattern was demonstrated between muscles in accordance with Kugelberg & Eklström (1968), Rosenberry & Kallnerth (1975). A 10 s pause in stimulation was found to result in a complete recovery in contractile strength of the gastrocnemii muscle. The soleus muscle on the other hand displayed a less pronounced recovery from fatigue. A more rapid restitution of ATP due to higher activity of creatine phosphokinase (Burke & Edgerton 1975) in the fast contracting gastrocnemii muscle was suggested to explain in part the discrepancy in recovery pattern. In preliminary experiments using the fatigue test described here a rapid restitution of contractile tension was observed during the first 15 s of recovery from exercise in spite of unchanged or slightly decreased lactate levels. Due to limited number of subjects studied, the influence of fibre type distribution could not be elucidated.

#### Central versus peripheral fatigue

"Muscle fatigue" is a complex phenomenon with numerous possible sites of origin.

"Central fatigue" in contrast to "peripheral fatigue" was suggested already in 1903 by Setchenov and even though Bigland-Ritchie et al. (1978) calculated that central fatigue may account for 30% of total force loss in sustained isometric contractions of the human quadriceps muscle the phenomenon was not observed during the initial phase of contraction. Hence force produced through voluntary contractions and contractions stimulated via the femoral nerve decreased to a similar extent during the first 30 s of contraction. One cannot preclude that changes in the "milieu interne" of the activated muscle via sensory receptors and their afferents will result in a central inhibition as has been suggested (e.g. Reid 1928, Lippold et al. 1960, Ammonson & Muxin 1978a). When again considering experiments originally described by Setchenov (1903), Ammonson & Muxin (1978a, b) it was demonstrated that exhausted individuals in a second bout of exercise were able to perform more work if diverting mental or physical activity were introduced in the pause instead of only rest. The observation was considered to be due to a change in the balance between the outflow



of inhibitory and facilitatory impulses to the central nervous system implicating that central inhibition might influence mechanical output during voluntary contraction

Whether or not central fatigue occurs failure at or distal to the neuromuscular junction must be considered mainly to limit the extent of voluntary contraction. Thus muscle contraction impairment may arise from failure of transmission at the neuromuscular junction action potentials which fail to propagate or from disturbances in calcium uptake release or binding capacity due to internal metabolic changes concomitantly affecting the coupling processes

#### SUMMARY

- 1 Physically active young men were studied in terms of muscle fibre type distribution in the vastus lateralis muscle and performance capacity during various types of short term exercise. The percentage of fast twitch (FT) muscle fibres averaged 49 (range 10-79) % in the studied muscle
- 2 Muscle force (torque) was recorded during repeated isokinetic knee extensions. The absolute as well as relative torque decline was taken as criterion for muscle fatigue and was found to be more pronounced in individuals with muscles rich in FT and/or FTb muscle fibres
- 3 Electromyography (EMG) was recorded from the vastus lateralis using surface electrodes. It was analysed for integrated EMG (IEMG) and power spectral density function (PSDF). In contrast to IEMG a general decrease in mean power frequency (MPF) was observed during repeated muscle contractions. This shift was due to a decrease in the high frequency bandwidth (associated with FT motor units) of EMG with a simultaneous increase in the bandwidth of low frequency components. In individuals characterized by a high percentage of FT fibres MPF decreased to a higher extent as compared to individuals with a high percentage of ST fibres
- 4 Lactate concentration and the activity of LDH<sub>tot</sub> and M-LDH were analysed in pools of different muscle fibre types. After various types of exercise (25 isokinetic contractions, supracardiac cycling exercise, downhill skiing) lactate concentration was related to % FT fibres in the activated muscle. Lactate concentration was also related to LDH<sub>tot</sub> and M-LDH activity. This seems to indicate a higher rate of lactate production in FT fibres as compared to ST fibres
- 5 After 25 maximal repeated contractions a relationship between force decline and FT/ST lactate ratio was demonstrated. A similar relationship was also demonstrated for LDH<sub>tot</sub> and M-LDH ratios indicating a close relationship between the metabolic profile of the different fibre types, muscle metabolism and muscle function

- 6 Lactate accumulation and associated metabolic changes interfering with the contractile mechanism is suggested to be responsible for muscle fatigue in the situations described here.

#### ACKNOWLEDGEMENTS

This work was carried out at the Department of Physiology III at Gymnastik- och idrottshögskolan Karolinska Institute Stockholm, directed by Professor Per-Olof Astrand, and at the Laboratory for Human Performance National Defence Research Institute Stockholm, directed by Professor Charles H Strömblad.

I wish to express my gratitude to Dr Jan Karlsson who introduced me to the field of exercise physiology; for guidance and enthusiastic support throughout the investigation. I am also indebted to Professor Lennart Kaijser and Dr Bertil Sjödin for valuable advice and constructive criticism.

My sincere thanks also to Miss Gurlith Petersson for excellent and patient secretarial help to Mrs Kerstin Elwin and Mrs Berit Sjöberg for skilful technical assistance and to my colleagues and co-workers who contributed to the stimulating atmosphere.

I wish to express my appreciation to all volunteers who participated in the studies.

This investigation was supported by grants from the Swedish Medical Research Council (No. 4251) and from the Research Council of the Swedish Sports Federation.

## REFERENCES

- ANDERSEN P 1975 Capillary density in skeletal muscle of man *Acta Physiol Scand* 95:203-205
- ANDERSEN P & SJØGAARD ■ 1976 Selective glycogen depletion in the subgroups of type II muscle fibres during intense submaximal exercise in man *Acta Physiol Scand* 96:26A.
- ANDERSEN P & BENRICKSSON J 1977 Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise *J Physiol* 270:677-690
- ASMUSSEN E, DÖRFLIN von W & HEDGSEN M 1948 Blood lactate and oxygen debt after exhaustive work at different oxygen tensions *Acta Physiol Scand* 15:57-62
- ASMUSSEN E & MAXIN B 1978a Recuporation after muscular fatigue by "Diverting Activities" *Eur J Appl Physiol* 38:1-7
- ASMUSSEN E & MAXIN ■ 1978b A central nervous component in local muscular fatigue *Eur J Appl Physiol* 38:9-15
- ASTRAND P-O & SAUTIN B 1961 Oxygen uptake during the first minutes of heavy muscular exercise. *J Appl Physiol* 16:971-976
- BAUDWIN R.M. & TIPTON C.M. 1972 Work and metabolic patterns of fast and slow twitch skeletal muscle contracting in situ *Pflügers Arch* 334:345-356
- BANG O 1936 The lactate content of the blood during and after muscular exercise in man *Scand Archiv für Physiologie Suppl* 10 74:51-82
- BARNY M. 1967 ATPase activity of myosin correlated with speed of muscle shortening *J gen Physiol* 50:197-218
- BARNY M. & CLOSE R.I 1971 The transformation of myosin in cross-innervated rat muscles *J Physiol (Lond.)* 213 455-474
- BARTLEY S. & CHUTE, R. 1947 *Fatigue and impairment in man* McGraw-Hill Book Company Inc New York
- BARTLEY ■ ■ 1965 *Fatigue: Mechanism and management* Charles C Thomas Publisher Springfield Illinois
- BENNETT C.H., PETERSON R.D & BOCKE R.M. 1963 Metabolism of red and white muscle fiber groups *Am J Physiol* 204(5) 939-942
- BENDAT J.S. & PIERCE, A.G 1971 *Random data: Analysis and measurement procedures* John Wiley & Sons New York
- BERGSTROM, J 1962 Muscle electrolytes in man *Scand J clin Lab Invest* Suppl 68
- BERGSTROM, J, HARRIS R.C., HULTMAN E. & MØRDESEN L-O 1971 Energy rich phosphagens in dynamic and static work *Adv Exp Med Biol* 11:341-355

- HIGLAND-RITCHIE B & WOODS J J 1974 Integrated EMG and oxygen uptake during dynamic contractions of human muscles *J Appl Physiol* 36:475-479
- HIGLAND-RITCHIE B, JONES D.A, HOSKING G.P & EDWARDS R.H T 1978 Central and peripheral fatigue in sustained maximum voluntary contractions of human quadriceps muscle *Clin Sci Mol Med* 54:609-614
- HOLSTAD G. & ENGLAND A. 1978 Energy metabolism in different human skeletal muscles during voluntary isometric contractions *Eur J Appl Physiol* 38 171-179
- BRADAL, P INQUIER, F & HENRIKSEN L 1977 Capillary supply of skeletal muscle fibers in untrained and endurance-trained man *Am J Physiol* 232(6):H705-H712
- BROOKE, M.H & KAISER, K.K 1970 Three "myosin adenosine triphosphatase systems: The nature of their pH lability and sulfhydryl dependence *J Histochem Cytochem* 18:670-672
- BROWN, G.L. & HORNE B B 1949 Fatigue and neuromuscular block in mammalian skeletal muscle *Proc Roy Soc B (Lond.)* 136:182-195
- BURKE, R.E. LEVINE D.B SAJAC P.E. TSAIRIS P & ENGEL W.K. 1971 Mammalian motor units: Physiological-histochemical correlation in three types in cat gastrocnemius *Science* N Y 174:709-712
- BURKE R.E. & EDEGGERON V R. 1975 Motor unit properties and selective involvement in movement. In: *Exercise and sport sciences reviews* pp 31-61 Vol. 3 Academic Press New York.
- CLOSE R.I 1972 Dynamic properties of mammalian skeletal muscles *Physiological Reviews* 52:129-197
- COBB S & FORBES A. 1923 Electromyographic studies of muscular fatigue in man *Am J Physiol* 65 234-251
- COATE E.F COSTILL, D.L. & LEBMES G.R. 1979 Leg extension power and muscle fiber composition. *Med Sci Sports* 11:12-15
- DANFORTH, W H. 1965 Activation of glycolytic pathway in muscle In: *Control of energy metabolism* (ed B. Chance et al ) pp 287-297 Academic Press New York.
- DIETS A.A. & LIEBOWITZ T 1967 Separation and quantitation of lactate dehydrogenase isoenzymes by disc electrophoresis *Analyt Biochem* 20 246-257
- DOMINOS J 1961 The metabolism of the tonic and tetanic muscles I Glycolytic metabolism. *Arch Biochem and Biophysics* 95:138-143
- ERENSTEIN A. & SANDOW A. 1961 Fatigue in phasic and tonic fibers of frog muscle *Science* 11 283-284
- ERENSTEIN A. & SANDOW A. 1963 Fatigue mechanisms in muscle fibres In: *The effect of use and disuse of neuromuscular functions* (ed. E. Gutzmann and P. Hnik) pp 515-526 Elsevier Publishing Company Amsterdam.

- EDGERTON V.R. SMITH J.L. & SIMPSON D.R. 1975 Muscle fibre type populations of human leg muscles. *Histochemical Journal* 7:259-266
- EDSTRÖM, L. & EKHOLM, B. 1972 Differences in sizes of red and white muscle fibres in vastus lateralis of musculus quadriceps femoris of normal individuals and athletes. Relation to physical performance. *Scand J clin Lab Invest* 30:175-181
- EDWARDS R.G. & LIPPOLD O.C.J. 1956 The relation between force and integrated electrical activity in fatigued muscle. *J Physiol* 132:677-681
- ENGEL W.K. 1962 The essentiality of histo- and cytochemical studies of skeletal muscle in the investigation of neuromuscular disease. *Neurology (Minneapolis)* 12:778-794
- ERIKSSON A. EKHOLM, J. HULTEN B. KARLSSON E. & KARLSSON J. 1976 Anatomical, histological and physiological factors in experienced downhill skiers. *Orthopedic Clinics of North America* 7:159-165
- ESSEN B. & HÄGGGÅRD T. 1975 Lactate concentration in type I and II muscle fibres during muscular contraction in man. *Acta Physiol Scand* 95:344-346
- ESSEN B. JONSSON E. HENRIKSSON J. TAYLOR, A.W. & SALATIN B. 1975a Metabolic characteristics of fibre types in human skeletal muscle. *Acta Physiol Scand* 95: 153-165
- ESSEN B. PERROW B. GOLLAUCK P.D. & SALATIN B. 1975b Muscle glycogen content and lactate uptake in exercising muscles. In: *Metabolic adaptation to prolonged physical exercise* (ed H. Rowald and J.R. Poortmans) pp 130-134. Birkhäuser Verlag Basel.
- ESSEN B. 1978 Studies on the regulation of metabolism in human skeletal muscle using intermittent exercise as an experimental model. *Acta Physiol Scand Suppl.* 454
- FITTS R.E. & HOLLOSZY J.O. 1976 Lactate and contractile forces in frog muscle during development of fatigue and recovery. *Am J Physiol* 231(2):430-433
- FITTS R.E. & HOLLOSZY J.O. 1977 Contractile properties of rat soleus muscle: effects of training and fatigue. *Am J Physiol* 233(3):C86-C91
- FITTS R.E. & HOLLOSZY J.O. 1978 Effects of fatigue and recovery on contractile properties of frog muscle. *J Appl Physiol:Respirat Environ Exercise Physiol* 45(6): 899-902
- FLETCHER, W.M. & HOPKINS P.G. 1907 Lactic acid in amphibian muscle. *J Physiol (Lond)* 35:247-250
- FLETCHER, W.M. 1914 Lactic acid formation, survival respiration and rigor mortis in mammalian muscle. *J Physiol* 47:361-380
- FOLDVÁR B. & HALICHA, H.D. 1968 A comparison between "red" and "white" muscle with respect to blood supply, capillary surface area and oxygen uptake during rest and exercise. *Microvascular Research* 1:1-14

- FUCHS P REDDY Y & BRIGGS F.W 1970 The interaction of cations with the calcium-binding site of troponin. *Biochim Biophys Acta* 221:407-409
- GOLDSPIKE, H LARSON R.E. & DAVIES R.E. 1970 The immediate energy supply and the cost of maintenance of isometric tension for different muscles in the hamster. *J vergl Physiol* 66:389-397
- GOLLRICK, P.D PIEHL K. SAUBERT IV C.W ARMSTRONG R.B & SALTIN B 1972a Diet, exercise and glycogen changes in human muscle fibers. *J Appl Physiol* 33(4) 421-425
- GOLLRICK P.D ARMSTRONG R.B SAUBERT IV C.W PIEHL K. & SALTIN B 1972b Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. *J Appl Physiol* 33(3):312-319
- GOLLRICK, P.D ARMSTRONG R.B SEMERONICH W.L. SHEPHERD R.E & SALTIN B 1973a Glycogen depletion pattern in human skeletal muscle fibers after heavy exercise. *J Appl Physiol* 34(5) 615-618
- GOLLRICK, P.D ARMSTRONG R.B SAUBERT IV C.W SEMERONICH W.L. SHEPHERD R.E & SALTIN B. 1973b Glycogen depletion patterns in human skeletal muscle fibers during prolonged work. *Pflügers Arch* 344:1-12
- GOLLRICK, P.D PIEHL, K. & SALTIN B 1974a Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. *J Physiol (Lond.)* 241:45-57
- GOLLRICK, P.D KARLSSON J, PIEHL, K. & SALTIN B. 1974b Selective glycogen depletion in skeletal muscle fibres of man following sustained contractions. *J Physiol (Lond.)* 241:59-67
- GOLLRICK, P.D BJÖRN B. KARLSSON J JONSSON E & SALTIN B 1974c Human soleus muscle: A comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Arch* 348:247-253
- GONDEL, G. 1941 The distribution of phosphatases in normal organs and tissues. *J Cell Comp Physiol* 17 71-83
- GRAHAM, T ESKGAARD G. LILLGREN H. & SALTIN B 1978 HAD in muscle of man at rest and during exercise. *Pflügers Arch* 376 35-39
- GRIMBY L. & HANSSON J 1977 Firing rate and recruitment order of toe extensor motor units in different modes of voluntary contraction. *J Physiol (Lond.)* 264 865-879
- GUDIKOV A. & KOSAROV D. 1974 Some features of different motor units in human biceps brachii. *Pflügers Arch* 347 75-88
- HOGSTADT, T JONSSON E. & SVANE, B. 1978 Cross-sectional area of the thigh muscle in man measured by computed tomography. *Scand J clin Lab Invest* 38 355-360
- HUMBERSTONE, C. & KILBICKI J -O 1975 Studies of some twitch and fatigue properties of different motor unit types in the ankle muscles of the adult cat. *Acta Physiol Scand* 95 231-242.

- SEUCHENOV I.M. 1935 Zur Frage nach der Eindringung sensitiver Reize auf di Muskelarbeit des Menschen. In: Selected works pp. 246-260 Moscow
- EDMONSON E 1971 Physiology of work capacity and fatigue Charles C Thomas Springfield Illinois
- SJÖDIN B 1976 Lactate dehydrogenase in human skeletal muscle Acta Physiol Scand Suppl. 436
- SPANDE J I & SCHOTTELINUS B A. 1970 Chemical basis of fatigue in isolated mouse soleus muscle Am J Physiol 219(5):1490-1495
- STÅLEBERG E 1966 Propagation velocity in human muscle fibers in situ Acta Physiol Scand Suppl 257
- STEPHENS J.A. & TAYLOR A. 1972 Fatigue of maintained voluntary muscle contraction in man. J Physiol (Lond.) 220:1-18
- STEPHENS J.A. & DEBENWOOD T P 1975 The fatigability of human motor units J Physiol 250:37-38P
- STRÖM, G. 1949 The influence of anaemia on lactate utilization in man after prolonged muscular work. Acta Physiol Scand 17:440-451
- TESCH, P & KARLSSON J 1977 Lactate in fast and slow twitch skeletal muscle fibres of man during isometric contraction Acta Physiol Scand 99:230-236
- THISTLE H.G. HISLOP H.J. MORFROID M. & LONGMAN E W 1967 Isokinetic contraction: a new concept of relative exercise Arch Phys Med Rehab 48:279-282
- THOMSON J.A. GREEN H.J & HOUSTON M.E. 1979 Muscle glycogen depletion patterns in fast twitch fibre subgroups of man during submaximal and supramaximal exercise Pflügers Arch 379:105-108
- THORSTENSSON A. 1976 Muscle strength, fibre types and enzyme activities in man Acta Physiol Scand Suppl 443
- THORSTENSSON A. & KARLSSON J 1976 Fatigability and fibre composition of human skeletal muscle Acta Physiol Scand 98:318-322
- THORSTENSSON A. SJÖDIN B TESCH P & KARLSSON J 1977 Actomyosin ATPase myokinase CPK and LDH in human fast and slow twitch muscle fibres Acta Physiol Scand 99:225-229
- WACHOLDER, K. 1931 Weitere Untersuchungen über das Verhalten "tonischer" und "nicht tonischer" Muskeln bei ermüdender Reizung Pflügers Arch 229:133 142
- WATKINS J R. & ENGEL W.K. 1972 Open biopsy electromyography Arch Neurol (Chic) 27:512-517
- VITASALO J H.T & KEMI P V 1975 Signal characteristics of EMG with special reference to reproducibility of measurements Acta Physiol Scand 93:531-539
- VITASALO J H.T & KEMI P V 1977 Signal characteristics of EMG during fatigue Eur J Appl Physiol 37:111-121





- SEITZHOV I.M. 1935 Zur Frage nach der Einwirkung sensitiver Reize auf die Muskelarbeit des Menschen. In: Selected works pp 246-260 Moscow
- SIMMONS E. 1971 Physiology of work capacity and fatigue Charles C. Thomas Springfield Illinois
- SÖDIN B. 1976 Lactate dehydrogenase in human skeletal muscle Acta Physiol Scand Suppl. 436
- SPARDE J I & SCHOTTELJUS H A. 1970 Chemical basis of fatigue in isolated mouse soleus muscle Am J Physiol 219(5):1490-1495
- STÅLBERG E. 1966 Propagation velocity in human muscle fibres in situ. Acta Physiol Scand Suppl. 287
- STEPHENS J.A. & TAYLOR, A. 1972 Fatigue of maintained voluntary muscle contraction in man. J Physiol (Lond.) 220:1-18
- STEPHENS J.A. & USHERWOOD T P. 1975 The fatigability of human motor units J Physiol 250:37-38P
- STRÖM, G. 1949 The influence of exercise on lactate utilization in man after prolonged muscular work Acta Physiol Scand 17:440-451
- TESCH P & KARLSSON J. 1977 Lactate in fast and slow twitch skeletal muscle fibres of man during isometric contraction Acta Physiol Scand 99:230-236
- THISTLE, H.G. HIDELOP H.J. MOFFROID M. & LOWMAN E.W. 1967 Isokinetic contraction: a new concept of resistive exercise Arch Phys Med Rehab 48:279-282
- THOMSON J.A. GREEN E J & HOUSTON M.E. 1979 Muscle glycogen depletion patterns in fast twitch fibre subgroups of man during submaximal and supramaximal exercise Pflügers Arch 379:105-108
- THORSTENSSON A. 1976 Muscle strength fibre types and enzyme activities in man. Acta Physiol Scand Suppl. 443
- THORSTENSSON A. & KARLSSON J. 1976 Fatigability and fibre composition of human skeletal muscle Acta Physiol Scand 98:316-322
- THORSTENSSON A. SÖDIN B. TESCH P & KARLSSON J. 1977 Actomyosin ATPase myo-kinase CPK and LDH in human fast and slow twitch muscle fibres Acta Physiol Scand 99:225-229
- WACHOLDER, K. 1931 Weitere Untersuchungen über das Verhalten "tonischer" und "nicht tonischer" Muskeln bei ermüdender Reizung Pflügers Arch 229:133-142
- WARMOLTS J.R. & ENGEL W.K. 1972 Open biopsy electromyography Arch Neurol (Chic) 27:512-517
- VIIITASALO J.H.T. & KEMI P.V. 1975 Signal characteristics of ENG with special reference to reproducibility of measurements Acta Physiol Scand 93:531-539
- VIIITASALO J.H.T. & KEMI P.V. 1977 Signal characteristics of ENG during fatigue Eur J Appl Physiol 37:111-121

ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 481

**SYMPOSIUM ON  
ION TRANSPORT  
ACROSS  
LIPID BILAYER MEMBRANES**

*Edited by*

*Ulrik V Lassen*

*Hans H Ussing      Jens Otto Wleth*

**COPENHAGEN 1980**



ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 481

**SYMPOSIUM ON  
ION TRANSPORT  
ACROSS  
LIPID BILAYER MEMBRANES**

*Edited by*

*Ulrik V Lassen*

*Hans H Ussing      Jens Otto Wleth*

**COPENHAGEN 1980**



ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 481

Symposium on  
ION TRANSPORT  
ACROSS LIPID BILAYER MEMBRANES

Edited by

*Ulrik V Lassen*

*Hans H Ussing      Jens Otto Wleth*

Copenhagen 1980



## Preface

The cell is the working unit of living organisms and each cell depends on regulatory transport functions of its plasma membrane. Lipids, arranged in a bi-molecular leaflet, constitute 50% or more of the building material of the cell membrane, but the properties of a hydrophobic lipid membrane cannot account for the permeabilities of biological membranes to hydrophilic molecules and ions. This was recognized and expressed by Danielli even before functional properties of artificial lipid membranes had been studied: "the membrane sandwich must contain active regions impermeable to most types of molecules through which selected ions or molecules can diffuse through the lipid barrier".

Artificial membranes play an important role for the endeavours to understand the transport properties of cell membranes. The general low permeability of unmodified lipid bilayers to ions and hydrophilic molecules was confirmed experimentally when Mueller & Rudin and Bangham showed that stable lipid bilayers—well suited for transport studies—can be formed *in vitro*. It was soon found that many characteristic features of the selective permeability properties of biological membranes are mimicked by modified artificial lipid membranes. The use of chemical compounds, which can induce selective permeabilities in artificial membranes, have thus become instrumental for understanding general physical and chemical principles of specialized transport processes. Identification and isolation of integral membrane proteins responsible for biological transport phenomena, have been fol-

lowed by promising attempts to "reconstitute" transport systems in artificial membranes. There is also substantial evidence that model systems will prove valuable for the investigation of interactions between membranes and membrane active hormones, toxins and drugs.

The present volume contains contributions from a symposium, Ion Transport across Lipid Bilayer Membranes, organized jointly by the Faculty of Natural Science and the Faculty of Medicine May 30, 1979 on occasion of the 500th anniversary of the University of Copenhagen. The chairman of the symposium was Professor D. C. Tosteson (Harvard Medical School Boston Mass. U.S.A.) who received an honorary degree as doctor of medicine at the anniversary celebrations in recognition of his important contributions to basic membrane research essential for the development of medical science.

The symposium was organized in close collaboration with Professor Tosteson. We feel that the invited speakers succeeded in their difficult task of presenting reviews of the major achievements in the field of lipid membrane research to an audience of scientists working mainly with other aspects of biological transport processes. It is the hope of the editors that the informative and stimulating presentations will reach a larger forum through this publication.

The symposium was made possible through a generous grant from the NOVO-Foundation.

*The Editors*





# Contents

## Preface 3

*D. C. Tosteson* (Department of Physiology, Harvard Medical School, Boston, USA) *On Bilayers and Biological Membranes* 7

*Beatrice M. Ann* (Department of Pharmacology, Ecole de Médecine, Geneva, Switzerland) *Reconstitution of the Na<sup>+</sup>/K<sup>+</sup> Transport System in Artificial Membranes* 15

*M. T. Tosteson* & *D. C. Tosteson* & *J. R. Benli* (Department of Physiology, Harvard Medical School, Boston, USA) *Cholera Toxin Interactions with Lipid Bilayers* 21

*Olaf Sparre Andersen* & *Joaquim Procopio* (Department of Physiology and Biophysics, Cornell University Medical College, New York, USA) *Ion Movement through a Gramicidin A Channel* 27

*Ramon Latorre* & *James J. Donovan* (Department of Physiology, Harvard Medical School, Boston, USA) *Modulation of A1 methiclin-Induced Conductance by Membrane Composition* 37

*R. Benz*, *D. C. C. K. Janke*, *P. Langer* & *G. St. rk* (Department of Biology, University of Konstanz, Konstanz, Germany): *Effects of Lipid Structure on the Kinetics of Carrier Mediated Ion Transport* 47



## On bilayers and biological membranes

B. C. TOSTESON

Department of Physiology Harvard Medical School, Boston, Mass. 02115 USA

It is now about 20 years since Mueller et al. (1963) introduced the technique of forming planar lipid bilayers between aqueous solutions. They suggested that this model system could be useful in exploring the physical chemistry of biological membranes. Scientists interested in the transport of ions, and particularly the electrical properties of biological membranes soon began to use the bilayer system. During the past decade many reports of such investigations have appeared in the literature (for recent reviews see Andersen (1978) Hall (1978) and Stark (1978)).

This paper is a brief and personal assessment of the significance of research on bilayers for our understanding of how ions move across biological membranes. I give special attention to two lines of work that have been pursued in our laboratories. One line is the role of the dipole potential in regulating the movements of ions across bilayers and biological membranes. The other line is the relation between the primary structure of peptides and decapeptides and their capacity to bind and promote transport of ions.

### *Some contributions of research on bilayers to ideas about ion transport across biological membranes*

In the 1940's when I began my work on the molecular mechanisms and cellular functions of ion transport across membranes, ion exchanger membranes were often put forward as a useful model (for example see Tosteson, 1955). However their thickness and composition were very different from those of biological membranes. The advent of planar lipid bilayers, particularly those containing little solvent (Montal & Mueller 1972), as well as closed lipid vesicles (Bangham, 1977) made available for the first time a model bearing considerable resemblance to the membranes present in living cells. One important impact of bilayer research then, has been to make concepts of ion transport in biology con-

siderably more realistic. For example measurements of the extremely low ion permeability of unmodified bilayers made of lipids extracted from biological membranes (Andreoli et al 1967a), contributed to the emergence of the notion that ion movements through biological membranes are mediated by proteins which penetrate through an otherwise largely impermeable liquid lipid matrix. With the increasing availability of pure membrane lipids, membrane-active compounds, and more recently membrane proteins, we are slowly step by step drawing a molecular picture of biological membranes and the ways that ions and other substances move through them.

Some of the most important ideas that have emerged in this process are illustrated by the program of this symposium. For example definite proof of the occurrence of ion conducting channels in bilayers through investigation of the effects of EIM, gramicidin, alamethicin and other compounds (see Hall 1978) have stimulated much interest among physiologists studying excitable membranes. These simple systems have permitted quantitative and precise characterization of ion selectivity, kinetics of ion movement and voltage dependence of channel opening and closing. As is made clear in other papers in this volume (Andersen 1980, Langer 1980, Latorre & Donovan 1980) one important lesson from these investigations is that even with very simple systems made with known chemical components many important questions about how ion conducting channels work remain unanswered. This experience provides a guide to the limits of useful speculation about ion transport through channels in biological membranes.

Another important use of bilayers in understanding biological membranes is in the reconstruction of biological transport systems. Because of the parallel arrangement of transport pathways in biological membranes, it is usually difficult or impossible to identify in the native membrane the class of mem-

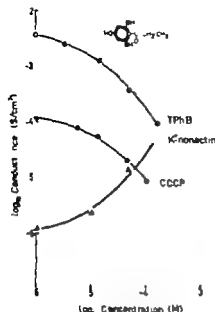


Fig. 1 The effect of 2,4,6-trihydroxypropiophenone on the conductance induced by  $\text{TPhB}^-$  (○), CCCP (●), and nonactin (Δ) in phosphatidylethanolamine membranes. In the TPhB and CCCP experiments the membranes were formed in 1 M NaCl, 10 mM  $\text{PO}_4$ , pH 6. TPhB was added to both sides of the membrane to a concentration of  $6.6 \times 10^{-6}$  M. CCCP was added to both sides of the membrane to a concentration of  $3.3 \times 10^{-6}$  M. In the nonactin experiment the membrane was formed in 1 M KCl, 10 mM  $\text{PO}_4$ , pH 6. Nonactin was added to a concentration of  $10^{-6}$  M. Temperature  $20 \pm 1^\circ\text{C}$ .

are membrane macromolecules responsible for a particular transport process. Isolation, separation and reconstitution in a bilayer devoid of extraneous components is therefore the most reliable route toward molecular definition of a biological transport system. In general vesicles have proved more useful than planar bilayers for reconstitution experiments (see for example Racker 1978) perhaps because the planar films used in most of the attempts at reconstitution have contained some hydrocarbon solvent. Notable achievements in reconstitution of biological transport systems include the Na-K pump (Goldin 1977), the Ca pump from sarcoplasmic reticulum (Racker & Eytan 1975), and the proton pump from purple bacteria (Racker & Stoekenius 1974) and the facilitated monovalent anion transport system found in mammalian red cells (Rothstein et al. 1975). In general the work reported to date has established the feasibility of reconstitution in bilayers of transport systems isolated from biological membranes but has

not yet contributed much to our understanding of how these molecular machines work. In this regard some of the investigations reported elsewhere in this volume (Anner 1980) give hope for the future.

Another promising direction of reconstitution experiments involves membrane receptors of hormones and drugs. Both the identification of such receptors and the impact of their interaction with agonists and antagonists on membrane properties, can be explored in bilayers. An example of this line of investigation is also present elsewhere in this volume (M. T. Tosteson et al. 1980).

Many results of research in bilayers with important implications for thinking about ion movements in biological membranes could not be represented elsewhere in this volume. The list includes the mechanism of action of uncouplers of oxidative phosphorylation (Lieberman et al. 1969; LeBlanc 1971), the significance for transport of chemical reactions involving transported substances in the relatively thick unstirred aqueous layers on the surface of membranes (Gutknecht et al. 1977), the role of surface charge in modifying the ionic composition of the water immediately adjacent to membranes and thus the transport of these ions across the membranes (McLaughlin et al. 1970), and the unequivocal demonstration of exchange diffusion in bilayers and a biological membrane (Wieth & Tosteson 1979; Tosteson & Wieth 1979). The remainder of this paper concerns two additional ideas emerging from investigations of bilayers.

#### *The role of the dipole potential in regulating ion transport across bilayers and biological membranes*

Lipid bilayers are several orders of magnitude more permeable to lipid soluble anions than to lipid soluble cations of comparable size and shape (Lieberman & Topaly 1969; LeBlanc 1970). Furthermore the surface potential produced at air-water interfaces by monolayers of phospholipid is oriented in the direction that the hydrophobic air-facing surface is positive relative to the underlying aqueous phase (Davies & Rideal 1963). These facts led Lieberman & Topaly (1969) and LeBlanc (1970) to suggest that the electrostatic potential in the hydrophobic interior of bilayers is positive relative to the membrane surfaces. This effect presumably arises from the interaction between the hydrophobic head groups of the membrane forming molecules, particularly the carbonyl oxygen atoms in the ester

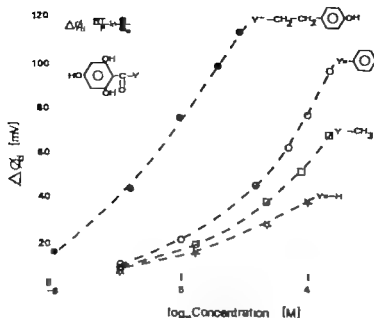


Fig. 2 The change in dipole potential ( $\Delta\phi_d$ ) promoted by different phloretin analogues.  $\Delta\phi_d$  was calculated on the basis of the equation given in the upper left corner of the Figure.  $\phi$  is the conductance of the membrane at a given analogue concentration in the aqueous phases and  $\phi_0$  is the conductance of the unmodified membrane. Points are referred to TPbB-conductance changes. Similar results are obtained with other ionic probes.

bonds of phospholipids with water dipoles in the adjacent aqueous solutions and hence has been called the dipole potential (Haydon & Myers 1973). Cass, Andersen and their colleagues (1973) and Andersen *et al.* (1976) observed that phloretin, the inhibitor of glucose and facilitated anion transport in red cells and other systems, increases cation but decreases anion permeability in bilayers. They suggested that phloretin produces these effects by reducing that is making less positive the dipole potential. We have confirmed and extended their observations (Melnik *et al.* 1977).


Coursin & Motals (1978) have recently observed that a series of phloretin analogues inhibit facilitated anion transport in red cells with differing potencies. They have attempted to rationalize their results in terms of the intrinsic dipole moment and hydrophobicity of the compounds. We have recently observed the effect of these compounds on the dipole potential in lipid bilayers (Latorre *et al.* 1979). Table 1 shows the structures of the compounds that we have studied. The intrinsic dipole moment of the compounds was varied by altering the number and position of -OH residues on the

aromatic ring. The hydrophobicity was varied by changing the substituents adjacent to the carbonyl group.

An example of the effect of one of these compounds, 2, 4, 6-trihydroxypropiophenone, on the conductance of bilayers to lipid soluble anions and cations is shown in Fig. 1. The conductance of bilayers exposed to the lipid soluble anions tetraphenyl boron and  $\text{CCCP}^-$  decreased while the conductance of bilayers exposed to the lipid soluble cations complex III-nonactin increased as the concentration of the propiophenone increased. The magnitude of the change in the dipole potential of the membrane can be estimated from the change in conductance for anions and/or for cations. A graph of the change in dipole potential as a function of the concentration of phloretin and several of the analogues that we have studied is shown in Fig. 2.

Using this approach we estimated the reduction in dipole potential produced by various concentrations of compounds shown in Table 1. From these data, we calculated the magnitude of the change in dipole potential ( $\Delta\phi_d$  in millivolts (mV)) in bilayers produced by the concentration required

Table 1



X	Y	$\mu$ (Debye)	PE (mV)	PE (mV)
2,4,6 Me		7	30	
2,4,6 Me	-Cl	1.3	21	<25
2,4,6 Me	-O-	1	10 <sup>2</sup>	30
2,4,6 Me	-O-C-	2	10 <sup>2</sup>	30
2,4,6 Me	-O-C-C-O-	2	10 <sup>2</sup>	25
2,4,6 Me	-O-	3	10 <sup>2</sup>	<25
2,4,6 Me	-O-	1.7	10 <sup>2</sup>	30
2,4,6 Me	-O-	1.3	10 <sup>2</sup>	30
2,4,6 Me	-O-	1.6	10 <sup>2</sup>	30
2,4,6 Me	-O-	1.7	10 <sup>2</sup>	<30

DATA FROM J. COUSIN AND R. MOTAS (1978) B B ACTA 857: 83-92.

to reduce the facilitated anion transport in beef red cells to one half of its maximum value (Cousin & Motas 1978). The results of these calculations are shown in Table 1. Note that the reduction in dipole potential was between 10 and 20 mV for most of the compounds studied.

These results raise the question of a correlation between the action of phloretin and other acetone derivatives on the dipole potential in bilayers and their capacity to inhibit facilitated transport systems in biological membranes. The relatively low specificity and potency of these compounds as inhibitors of biological membrane transport systems are consistent with the hypothesis that they exert their effect on some general property of membranes. From the expected reduction in positivity of the intra-membrane electrostatic potential it is reasonable to suspect that transport processes inhibited by these compounds share the property that the movement of negative charge across the membrane interior is rate-limiting. Another possible interpretation is that the large dipole potential difference is important in maintaining the shape and orientation of transport proteins in biological membranes. Clearly these two lines of speculation are not mutually exclusive.

In the light of these results it is interesting to ask about the role of local electrostatic potentials in the mechanism of action of various membrane agonists and antagonists. Most neurotransmitters and membrane active hormones have significant permanent

or induced dipole moments and many possess a formal electrical charge. Perhaps one role of membrane receptors is to fix and thus increase the local concentration of an agent with the capability of altering the local electrostatic fields and thus the position and mobility of ligands on nearby membrane proteins.

### *The relation between molecular structure and transport function of cyclic peptides and depsipeptides*

Valinomycin has long been known to react with monovalent cations to produce positively charged complexes which readily traverse biological membranes (Pressman 1965) and lipid bilayers (Mueflier & Rodin 1967; Lev & Buzhinsky 1967; Andreoli et al 1967b). Relatively minor changes in the primary structure of this cyclic dodecadepsipeptide profoundly alter its capacity to act as an ion carrier in membranes (Sternyak et al 1965). Many substitutions of single residues interfere with the formation of the six intra-molecular hydrogen bonds that stabilize the bracelet-shaped complex between valinomycin and cation. However certain analogues retain the capacity to form stable ion complexes and yet exhibit striking differences from the parent compound in their ability to promote ion transport across membranes. We have studied one such series of compounds that produced by substituting D or L proline for D isohydroxyvalerate and L lactate respectively (Glin & Merrifield 1972; Ting-Beall et al 1974; Davis et al 1976; Benz et al 1976; Glin et al 1978). The primary structures of these compounds are:

Valinomycin (val) cyclo (Dval-Llac-Lval-Dtyr)<sub>3</sub>  
 PV lac cyclo (Dval-Llac-Lval-Dpro)<sub>3</sub>  
 PV cyclo (Dval-Lpro-Lval-Dpro)<sub>3</sub>

These rather similar compounds differ markedly in their capacity to promote transport of ions across bilayers. For example the maximum current produced by a high electrical potential difference across glycerolmonooleate bilayers exposed to 10<sup>-6</sup> M cyclic peptide and 0.1 M KCl are about 3×10<sup>-6</sup>, 3×10<sup>-6</sup> and 10<sup>-6</sup> A cm for PV, PVlac and val respectively. Thus replacement of the three hydroxyvalerate residues in valinomycin by proline reduces the maximum current by a factor of 10<sup>2</sup> and the additional substitution of prolines for the lactate residues produces a further large reduction. Furthermore unilateral addition of cyclic pep-

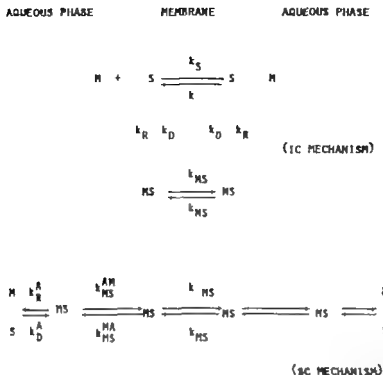


Fig. 3 Reaction scheme for the transport of an ion  $M$  by the carrier  $S$ . In the interfacial complexation mechanism (IC), the complex  $MS^+$  is formed at the membrane solution interface. In the solution complexation (SC) mechanism, the complex is formed in the aqueous phase.

tide to bilayers separating otherwise identical salt solutions (e.g. 0.1 M KCl) produces a steady state electrical potential difference in the case of PV but not for PVlac and val (Ting-Beall et al. 1974; Latorre & Korosbetz, unpublished data). Observations about the effect of these compounds on both the steady state and the kinetic characteristics of ion transport across bilayers can be interpreted in terms of the relatively simple model shown in Fig. 3.

The ion carrier  $S$  (one of the three cyclic peptides) can react with a metal ion  $M$  to form a positively charged complex,  $MS^+$ . This reaction can occur either in the aqueous solutions or as an interfacial reaction between  $M$  in the aqueous solution and  $S$  on the membrane surface. Both  $M$  and  $S$  can adsorb to the membrane as defined by the partition coefficients  $\beta_{MS}$  and  $\beta_S$  and translocate between the surfaces at rates determined by the differences in interfacial concentration and values of the rate coefficients  $k_{MS}$  and  $k_S$ . Because the complex  $MS^+$  is positively charged its movement is also affected by electrical potential differences between the membrane surfaces. Two general classes

of transport mechanism can be distinguished. In one type interfacial complexation the transported ion reacts with the cyclic peptide on the membrane surface. In the other type the transported ion reacts with the carrier in the aqueous solutions and moves through the unstirred layer as the complex which then adsorbs to and moves through the membrane. This solution complexation mechanism can occur only when the rate coefficients for the reaction  $k_R$  and  $k_D$ , and/or the rate coefficient for translocation of the free carrier  $k_A$  are too slow to permit the cycle necessary for the interfacial complexation mechanism to proceed sufficiently rapidly.

Values for the rate coefficients for translocation  $k_{MS}$  and  $k_S$ , the rate coefficients for the interfacial reaction  $k_R$  and  $k_D$ , the partition coefficients  $\beta_{MS}$  and  $\beta_S$  and the aqueous dissociation constant  $k_{MS}$  for the three compounds in glycerolmonoolate membranes are shown in Table 2. As shown clearly and convincingly by Luger and his colleagues (Stark et al. 1971) valinomycin operates as an ion carrier by the interfacial complexation mechanism and all four



Table 2. Rate constants for valinomycin-mediated and valinomycin analogs mediated K transport

	$k_a$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_b$ (s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	$k$ (s <sup>-1</sup> )	$\beta_a$ (cm)	$\beta_{cat}$ (cm)	$K$ (M <sup>-1</sup> )	REF
val	$3.0 \times 10^8$	$3 \times 10^8$	$2 \times 10^8$	$4.0 \times 10^4$	0.004	—	<0.1	b, c
PV Lac	$2.3 \times 10^8$	$4 \times 10^8$	$5 \times 10^4$	$5.5 \times 10^3$	0.0023	-0.007	<1.5	d
PV	—	—	$5 \times 10^3$	—	—	0.003	8	e
Glycophospholipid membranes								
(1976)	Stark & Benz (1971)		Benz & Langer (1976)		Present results		Benz et al.	

of the relevant rate coefficients have approximately the same value of  $10^8$  sec<sup>-1</sup>. PV Lac and PV function less well as ion carriers not because they fail to partition into the membrane or bind cations but rather because of their kinetic properties. In fact, the partition coefficient for the free form of PV Lac  $\beta_a$  is not significantly different from that for val while both PV Lac and PV have a much greater affinity than val for K<sup>+</sup> in water. The greater affinity of PV for cations is even more evident in nonaqueous solvents (Davis et al. 1976). The most striking difference between PV Lac and val is a reduction in the value for the rate coefficient for translocation of the free peptide  $k_a$  by a factor of almost three orders of magnitude. This value of  $k_a$  permits PV Lac to carry K<sup>+</sup> by the interfacial complexation mechanism but at a much slower rate in the steady state than val. For PV the values for  $k_a$ ,  $k_b$  and  $k_{cat}$  were too low to estimate by electric relaxation techniques and this compound promotes K<sup>+</sup> movement across bilayers by the solution complexation mechanism. Only  $k_{cat}$ , the rate coefficient for translocation of the ion complex, could be measured for all three compounds. For PV Lac it is four-fold and for PV forty-fold lower than for val.

It is interesting to inquire about the molecular basis for the impressive differences between these three cyclic dodecapeptides as ion carriers. On the basis of their proton nmr spectra it is highly probable that the shape and size of the K<sup>+</sup> complexes of val and PV are almost identical (David et al. 1976). Therefore the forty-fold reduction in  $k_{cat}$  for PV compared with val is probably not due to differences in the mobility of the complexes within the membrane. A more likely explanation is that the presence of the cyclic proline side chains rather than the isopropyl and methyl side chains of the hydroxy acids makes the hydrophobicity of the external surfaces of the PV K<sup>+</sup> complex different from that of the val-K<sup>+</sup> complex. PV Lac might be an intermediate case.

Of even greater significance for their effectiveness as ion carriers are the differences in  $k_a$ , the rate coefficient for translocation of the free form of PV Lac and val. Proton nmr experiments show clearly that the number of conformers of the free form of PV is greater than for val. Furthermore the rates of the transitions between these forms is slower for PV and for val (Davis et al. 1976; Grain et al. 1978). This result is not surprising given the relative rigidity of the proline residues. Indeed the slow rate of dissociation of the PV K<sup>+</sup> complex revealed in the nmr data probably derives from the same property of proline and is in large part responsible for the fact that PV promotes K<sup>+</sup> movement across bilayers by the solution rather than the interfacial complexation mechanism. Slow transitions between multiple conformational states of the free forms could explain the low values of  $k_a$  for the proline-containing compounds. The conformer released upon dissociation of the ion complex could be relatively hydrophilic and traverse the membrane only very slowly. The slow conversion to a more hydrophobic conformer could limit the rate of transport.

The analysis of these three cyclic peptides illustrates that relatively minor and subtle changes in primary structure can have profound and complicated effects on their properties as ion carriers. As we come to understand the primary structures of biological membrane transport proteins it is likely that similar complexities will become evident. Perhaps the lessons learned with these simple systems will be useful in addressing the problems of ion transport mediated by macromolecules.

This work was supported by grant GM 27715 from the National Institute of Health.

## REFERENCES

- ANDERSEN O. S. 1978. Permeability properties of unmodified lipid bilayer membranes. In *Membrane transport I: biology*. Vol. I. Concepts and models (ed.

- D. C. TOSTESON) pp 369-446 Springer-Verlag, Berlin Heidelberg, New York.
- ANDERSEN O. S. 1980 This Volume pp. 7-36.
- ANDERSEN O. S. FINKELSTEIN A. KATZ, I. & CASS A. 1976 Effects of phloretin on the permeability of thin lipid membranes. *J Gen Physiol* 67 749-771.
- ANDREOLI T. E. BANGHAM J. A. & TOSTESON D. C. 1967 a. The formation and properties of thin lipid membranes from HK and LR sheep red cell lipids. *J Gen Physiol* 50: 1729-1749.
- ANDREOLI T. E., TIEFFENBERG M. & TOSTESON D. C. 1976 b. The effect of valinomycin on the ionic permeability of thin lipid membranes. *J Gen Physiol* 50 2527-2545.
- ANNER, B. 1980 This Volume pp 15-70.
- BANGHAM A. D. 1972. Lipid bilayers and biomembranes. *Annu Rev Biochem* 41 753-776.
- BENZ, R. GISEN B. BEALL, P. T., TOSTESON D. C. & LAUGER P. 1976. Mechanism of ion transport through lipid bilayer membranes mediated by peptide cyclo-(D-Val-L-Pro-L-Val-D-Pro). *Biochim Biophys Acta* 455 655-685.
- CASS, A. ANDERSEN O. S. KATZ, J. & FINKELSTEIN A. 1973 Phloretin's action on cation and anion permeability of modified lipid bilayers. *Biophys Soc Abstr TAM-BIZ* 108a.
- COUSIN J. L. & MOTAIS R. 1978 Effect of phloretin on chloride permeability: A structure activity study. *Biochem Biophys Acta* 507 531-538.
- DAVIES J. T. & RIDEAL, E. K. 1963 Interfacial phenomena. 2nd ed. Academic Press, New York.
- DAVIS, D. G. GISEN J. B. & TOSTESON D. C. 1976. Conformational studies of Peptide PV cation-binding analogue of valinomycin. *Biochem* 15 768-774.
- GISEN B. F. & MERRIFIELD R. B. 1972. Synthesis of hydrophobic, potassium-binding proteins. *J Amer Chem Soc* 94 6165-6170.
- GISEN B. F. TING-BEALL, H. P. DAVIS D. G. GRELL, E. & TOSTESON D. C. 1978. Selective ion binding and membrane activity of synthetic cyclo-peptides. *Biochim Biophys Acta* 509: 201-217.
- GOLDIN S. M. 1977 Active transport of sodium and potassium ions by the sodium and potassium ion activated adenosine triphosphatase from renal medulla. reconstitution of the purified enzyme into well-defined *in vitro* transport system. *J Biol Chem* 252 5630-5642.
- OUTKNECHT J. BISSON M. A. & TOSTESON D. C. 1977 Diffusion of carbon dioxide through lipid bilayer membranes. *J Gen Physiol* 69: 779-794.
- HALL, J. H. 1978. Channels in black lipid films. In: *Membrane transport in biology Vol 1 Concepts and model* (ed D. C. Tosteson) pp. 476-531 Springer Verlag, Berlin, Heidelberg, New York.
- HAYDON P. A. & MYERS V. B. 1973 Surface charge, surface dipoles and membrane conductance. *Biochim Biophys Acta* 303 429-443.
- LATORRE, R. & DONOVAN J. J. 1980 This volume pp. 37-46.
- LATORRE R. REYES, J. MOTAIS R. & TOSTESON D. C. 1979 Effect of phloretin analogues on ion transport. *Biophys J* 258a.
- LAUGER, P. 1980 This Volume pp 47-52.
- LEBLANC O. H. Fr 1970 Single ion conductance in lipid bilayers. *Biophys Soc Ann Meet Abstr* 14.94a.
- LEBLANC O. H. Jr 1971 The effect of uncouplers of oxidative phosphorylation on lipid bilayer membranes: carbonylcyanide *m*-chlorophenyl hydrazone. *J Membr Biol* 4 227-251.
- LEV A. A. & BUZHINSKY E. P. 1967 Cation specificity of the model biomolecular phospholipid membranes with incorp valinomycin. *Tsitolgija* 9: 102-105.
- LIBERMAN YE. A. & TOPALY V. P. 1969 Permeability of biomolecular phospholipid membranes for fast soluble ions. *Biofizika* 14 452-461.
- LIBERMAN E. A. TOPALY V. P. TSOFINA, L. M., JASAITIS A. A. & SKULACHEV B. P. 1969 Mechanism of coupling of oxidative phosphorylation and membrane potential of mitochondria. *Nature* 222: 1076-1078.
- MCLAUGHLIN S. E. A. SZABO G. EISENMANN G. & CIANI S. M. 1970 Surface charge and the conductance of phospholipid membranes. *Proc Natl Acad Sci USA* 66 1268-1275.
- MELNIK, E., LATORRE, R., HALL, J. E. & TOSTESON D. C. 1977 Phloretin-induced changes in ion transport across lipid bilayer membranes. *J Gen Physiol* 69: 243-257.
- MONTAL, M. & MUELLER, P. 1972. Formation of biomolecular membranes from lipid monolayers and study of their electrical properties. *Proc Natl Acad Sci USA* 69: 3561-3566.
- MUELLER, P. RUDIN D. O. TITEN H. & WESCOTT W. C. 1963 Methods for the formation of single biomolecular lipid membranes in aqueous solution. *J Phys Chem* 67 543-550.
- MUELLER, P. & RUDIN D. O. 1967 Development of Na<sup>+</sup>-K<sup>+</sup> discrimination in experimental biomolecular lipid membranes by monocyclic antibodies. *Biochem Biophys Res Commun* 26: 398-404.
- PRESSMAN B. 1965 Induced active transport of ions in mitochondria. *Proc Natl Acad Sci* 53 1076-1083.
- RACKER, E. 1978. Mechanisms of ion transport and ATP formation. In: *Membrane transport in biology Vol 1 Concepts and model* (ed D. C. Tosteson) pp. 259-287 Springer Verlag, Berlin, Heidelberg New York.
- RACKER, E. & STOECKENIUS, W. 1974 Reconstruction of purple membrane vesicles catalyzing light-driven proton uptake & adenosine triphosphate formation. *J Biol Chem* 49: 662-666.
- RACKER, E. & EYTAN E. 1975 A coupling factor from sarcoplasmic reticulum required for the translocation of Ca<sup>2+</sup> ions in reconstructed Ca<sup>2+</sup> ATPase pump. *J Biol Chem* 250 7533-7536.
- ROTHSTEIN A. CABANTCHIK Z. L. BALSHIN M. & JULIANO R. 1975 Enhancement of anion permeability in lecithin vesicles by hydrophobic proteins traced from red blood cell membranes. *Biochem Biophys Res Commun* 64 144-150.
- SHEMYAKIN M. M. VINOGRADOVA, E. I., FEJGINA, M. Y., ALDANOVA, N. A., LOGINOVA, N. F. RYABOVA, I. P. & PAVLENKO L. A.

- 1965 The structure-antimicrobial relation of valinomycin depsipeptides. *Experientia* 21 548-552.
- STARK, G 1978. Carrier-mediated ion transport across thin lipid membranes. In: *Membrane transport in biology* Vol. I Concepts and models (ed. D C. Tosteson) pp 447-473. Springer Verlag Berlin, Heidelberg, New York.
- STARK, G, KETTERER, B, BENZ, R. & LÄUGER, H 1971. The rate constants of valinomycin-mediated ion transport through thin lipid membranes. *Biophys J* 11 981-994.
- TING-BEALL, H P, TOSTESON M T, GISEN B P & TOSTESON D C 1974. The effect of peptide PV on the ionic permeability of lipid bilayer membranes. *J Gen Physiol* 63 492-508.
- TOSTESON D C. 1955. Sodium and potassium transport in red blood cells. In: *Electrolytes in biological systems* (ed. A. M. Shoenes) p 123. Am Physiol Soc Washington.
- TOSTESON M T, RUBNITZ, J & TOSTESON D C. 1980. This Volume pp 21-26.
- TOSTESON M T & WIETH J O 1979. Tributyltin-mediated exchange diffusion of halides in lipid bilayers. *J Gen Physiol* 73 789-800.
- WIETH J O & TOSTESON M. T 1979. Organotin-mediated exchange diffusion of anions in human red cells. *J Gen Physiol* 73 765-788.

## Reconstitution of the $\text{Na}^+ \text{K}^+$ transport system In artificial membranes

BEATRICE M. ANNER

Department of Pharmacology, Ecole de Médecine, CH 1211 Geneva 4, Switzerland

ANNER, B. M. Reconstitution of the  $\text{Na}^+ \text{K}^+$ -transport system in artificial membranes. *Acta Physiol Scand* 1980, Suppl. 481 15-19. Department of Pharmacology, Ecole de Médecine, Geneva, Switzerland.

(1) Purified renal  $\text{Na}^+ \text{K}^+$ -ATPase was incorporated into phosphatidylcholine-liposomes by using the cholato-dialysis procedure. The passive permeability of the liposomes for  $\text{Na}^+$  and  $\text{K}^+$  was determined on  $^{22}\text{Na}$  and  $^{86}\text{Rb}$  equilibration curves. The rate constant for passive  $\text{Na}$ -flux was  $0.014 \text{ min}^{-1}$  and for  $\text{K}$  ( $^{86}\text{Rb}$ )-flux  $0.013 \text{ min}^{-1}$ . Active transport rates of  $\text{Na}^+$  and  $\text{K}^+$  were determined after addition of  $3 \text{ mM}$  ATP to the liposomes. (2) A micro-method for continuously measuring the ADP production associated with active transport is described. Using this technique initial velocities of ATP hydrolysis were measured in parallel with  $\text{Na}^+$ -transport and  $\text{K}^+$ -transport. When the  $\text{Na}^+$ -concentration in the incubation medium is increased, the initial velocity of all three processes increase concomitantly. At high turnover the stoichiometry of coupled  $\text{Na}^+ \text{K}^+$ -transport catalyzed by the purified reconstituted pump is  $2.2\text{Na}^+ : 1\text{K}^+ : 1\text{ATP}$ . (3) When the pump has exhausted the vesicular  $\text{K}^+$ -pool, coupled  $\text{Na}^+ \text{K}^+$ -transport ceases. The pump then carries  $\text{Na}^+$  uphill without  $\text{K}^+$ -export. The  $\text{Na}^+/\text{ATP}$  ratio of uncoupled  $\text{Na}^+$ -transport is close to 1.

**Key word:** Reconstituted  $\text{Na}^+ \text{K}^+$ -pump stoichiometry uncoupled  $\text{Na}^+$ -transport

Most living cells expell sodium ions and accumulate potassium ions. This coupled transport process is mediated by an ATP-driven pump located in the cell membrane. The stoichiometry of the  $\text{Na}^+ \text{K}^+$  pump in red blood cells and in excitable tissues is around  $3\text{Na}^+ : 2\text{K}^+ : 1\text{ATP}$  (for a review of the sodium pump see Glynn & Karlish 1975).

From early work on crude  $\text{Na}^+ \text{K}^+$ -ATPase from peripheral nerve (Skou 1957) and from later work on the purified enzyme it became very probable that  $\text{Na}^+ \text{K}^+$ -ATPase was the  $\text{Na}^+ \text{K}^+$  transport system (for recent reviews of the  $\text{Na}^+ \text{K}^+$ -ATPase see Schwartz et al 1975, Jørgensen 1975, Skou 1975). This was confirmed by the important demonstration that  $\text{Na}^+ \text{K}^+$ -ATPase reconstituted in liposomes catalyzed ATP-dependent  $\text{Na}^+$ -transport (Goldin & Tong 1974, Hilden et al 1974, Racker & Fløe 1975).

There is much circumstantial evidence that the

$\text{Na}^+ \text{K}^+$ -pump is also responsible for transepithelial  $\text{Na}^+$ -transport (Koefoed-Johnsen & Ussing 1958, DiBona & Mills 1979). The observation that  $\text{Na}^+ \text{K}^+$ -ATPase from dogfish rectal salt gland (Hilden & Hokin 1975) or from renal outer medulla (Anner et al 1976, 1977) catalyzes coupled active  $\text{Na}^+ \text{K}^+$  transport after reconstitution in liposomes supports this concept. Moreover it has been observed that the  $\text{Na}^+$ -transport capacity of purified  $\text{Na}^+ \text{K}^+$ -ATPase appears to be flexible to some extent (Anner et al 1977). This finding renders the role of the  $\text{Na}^+ \text{K}^+$ -pump in transepithelial transport even more plausible.

In the present work the nature of the additional  $\text{Na}^+$  transport component was investigated. To that end, initial transport velocities,  $\text{Na}^+$ - $\text{K}^+$ -gradients and the ATP hydrolysis associated with transport were measured. These combined methods have allowed to determine the coupling ratio of the

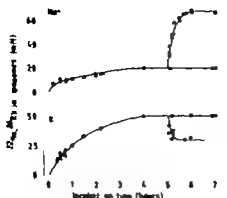


Fig. 1 Time course (O) for loading of liposomes with  $^{22}\text{Na}$  and  $^{86}\text{Rb}$ . Liposomes were formed in solution A containing 20 mM NaCl and 50 mM KCl and were incubated for 60 min at 25°C. At zero time on the abscissa, tracer was added and the isotope content of the liposomes was measured after removal of free isotope by gel filtration on Sephadex G-50 medium columns and calculated as a fraction of the vesicular isotope content at equilibrium. Active transport (●) was then initiated by adding 3 mM Tris-ATP to aliquots of the liposome suspension. The ATPase activity of free enzyme was inhibited with 1 mM ouabain when the active transport assay was started. Net active cation transport was calculated from the difference of the radioactivity between liposomes incubated with ATP and control liposomes incubated without ATP.

Na<sup>+</sup>/K<sup>+</sup>-exchange and iii) confirm that a fraction of the active net Na<sup>+</sup>-transport is not coupled to K<sup>+</sup>-transport.

## METHODS

Na<sup>+</sup>/K<sup>+</sup> ATPase from the outer medulla of rabbit kidney was purified by the single rotor procedure described by Jørgensen (1974). Protein was determined according to Lowry et al. (1951) in the presence of 0.1% sodium dodecyl sulfate.

For solubilization, 1 mg Na<sup>+</sup>/K<sup>+</sup> ATPase protein with a specific activity of 800–1200 µmoles P/mg protein/h was sedimented at 120 000 g for 180 min and the pellet was resuspended in 100 µl of solution A: choline chloride 50 mM, L-cysteine 1 mM, EDTA 1 mM, MgCl<sub>2</sub> 5 mM, varying concentrations of NaCl and KCl, mizudazole 30 mM, pH 7.10 and in addition 1% sodium cholate. The undissolved enzyme was sedimented at 100 000 g for 15 min.

After addition of phosphatidylcholine to the soluble enzyme, liposomes were formed by dialysis and active transport was measured as described before (Anner & Jørgensen 1979) except that 1 mM ouabain was present during the active transport assay.

The transport-associated ATP hydrolysis was measured by a modification of the linked-enzyme assay (Schwartz et al. 1971) and the time course was continuously recorded. A 500 µl continuous flow quartz-cuvette was placed in a

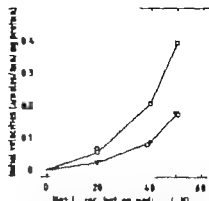


Fig. 2 Initial velocities of Na<sup>+</sup>-transport (□), K<sup>+</sup>-transport (△) and ATP-hydrolysis (○) as a function of the Na<sup>+</sup>-concentration in the incubation medium. Liposomes were prepared in solution A containing 20, 40 or 50 mM NaCl. KCl was kept constant at 50 mM. Initial velocities were measured between 30 and 300 s after ATP addition. The change of the isotope content was calculated as described in the Legend for Fig. 1. The initial velocity of transport-associated ATP-hydrolysis was read on recordings obtained as described under Methods.

Uvicam SP 1800 spectrophotometer through a thin tubing with 490–495 µl of solution A containing in addition Tris-ATP 3 mM, ouabain 0.2 mM, NADH 0.1 mM, phosphoenolpyruvate 1.3 mM and 4 µl of a combined pyruvate kinase/lactate dehydrogenase suspension (Boehringer). The baseline was recorded for 20 min at 25°C. Then 5–10 µl of the liposomes suspension were added and the tubing was rapidly rinsed with a portion of the cuvette solution. The ATPase activity of free enzyme was negligible in the presence of 0.2 mM ouabain. The initial velocity of the ATP hydrolysis as well as the succeeding non linear part and the final linear part at steady state were recorded.

## RESULTS

### Active and passive fluxes of Na<sup>+</sup> and K<sup>+</sup> in liposomes

For the study shown in Fig. 1 the liposomes were prepared in a solution containing 20 mM NaCl and 50 mM KCl. The suspension was then incubated at 25°C and equilibrated with  $^{22}\text{Na}$  or  $^{86}\text{Rb}$  within 4 to 5 hours. The rate constant for passive flux was 0.014 min<sup>-1</sup> for K<sup>+</sup> and 0.013 min<sup>-1</sup> for Na<sup>+</sup>.

When ATP was added to the equilibrated liposomes the vesicular Na<sup>+</sup> increased 2.9 fold whereas the vesicular K<sup>+</sup> fell to 60% of the initial K<sup>+</sup> content. Calculated on the total vesicle pool the Na<sup>+</sup>-gradient was 48 mM and the K<sup>+</sup> gradient 20 mM at steady state. Although active Na<sup>+</sup>/K<sup>+</sup>

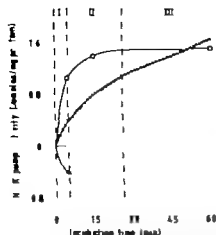


Fig. 3 Time course of active  $\text{Na}^+$ -transport (O),  $\text{K}^+$ -transport (V) and ATP hydrolysis (■). Liposomes were prepared in solution A containing 40 mM NaCl and 50 mM KCl. Net active cation transport was started by adding 3 mM Tris-ATP to the liposomes at isotopic equilibrium. Active cation transport was measured as explained in Fig. 1. The ATP hydrolysis curve was plotted from recording obtained as described under Methods. Phase I was delimited when  $\text{K}^+$ -transport reached a plateau and phase II when  $\text{Na}^+$ -transport reached a plateau. Phase III designed  $\text{Na}^+$   $\text{K}^+$ -gradients at steady state.

transport proceeded slowly when the incubation medium contained 20 mM  $\text{Na}^+$  and 50 mM  $\text{K}^+$ . (See Fig. 2) the rate constant of active flux were 7–10 times larger than for passive  $\text{Na}^+$  and  $\text{K}^+$  fluxes. The pronounced difference of the passive and active  $\text{Na}^+$   $\text{K}^+$  fluxes allows the precise determination of the initial velocity of active transport in phosphatidylcholine-liposomes.

#### The stoichiometry of the ATP-dependent $\text{Na}^+$ $\text{K}^+$ -transport

The initial velocity of  $\text{Na}^+$ -transport,  $\text{K}^+$ -transport and ATP-hydrolysis was determined at 20, 40 and 50 mM  $\text{Na}^+$  while  $\text{K}^+$  was kept constant at 40 mM. Fig. 3 illustrates that at 50 mM  $\text{Na}^+$  the initial velocity was 8 times larger for  $\text{Na}^+$ -transport, 7 times larger for  $\text{K}^+$ -transport and 3.5 times larger for ATP hydrolysis as compared to the velocities measured at 20 mM  $\text{Na}^+$ . When the rates at 50 mM  $\text{Na}^+$  were compared to the rates obtained at 40 mM  $\text{Na}^+$  the increase was close to 2 for all three processes. Consequently the  $\text{Na}^+$   $\text{K}^+$  coupling was rigid despite a 8 fold increase of the initial transport velocity. The average coupling ratio was  $2.2 \text{ Na}^+ : 1 \text{ K}^+$ .

ATP hydrolysis was tightly coupled to active  $\text{Na}^+$   $\text{K}^+$ -transport at 40 and 50 mM  $\text{Na}^+$  and the ratio was  $2.2 \text{ Na}^+ : 1 \text{ K}^+ : 1 \text{ ATP}$ . However at 20 mM  $\text{Na}^+$  where the transport velocity was low (Fig. 2) the number of ATP molecules hydrolyzed was equal or higher than the number of  $\text{Na}^+$  transported. This would imply that increasing  $\text{Na}^+$  in the incubation medium from 20 to 40 mM or 50 mM activates a  $\text{Na}^+$  site which not only stimulates initial transport velocities, but also facilitates the coupling between ATP hydrolysis and active  $\text{Na}^+$   $\text{K}^+$ -transport. Yet, a more detailed analysis of this specific problem is necessary to consolidate this observation. The existence of multiple  $\text{Na}^+$  sites appears also from the initial sigmoidal relationship between transport velocity and  $\text{Na}^+$  concentrations (Fig. 2).

#### Evidence for active uncoupled $\text{Na}^+$ -transport

From the experiments described above it appears that the  $\text{Na}^+$  and  $\text{K}^+$ -transport is tightly coupled. This implies that  $\text{Na}^+$ -transport should cease as soon as the pump has depleted the liposomes of  $\text{K}^+$ . Yet above a limiting  $\text{Na}^+$ -concentration in the incubation medium, net  $\text{Na}^+$  transport continues. The  $\text{Na}^+$   $\text{K}^+$ -pump appears to switch to uncoupled  $\text{Na}^+$ -transport as soon as the concentration at the  $\text{K}^+$  site has reached a low limiting level. The exact  $\text{Na}^+$  requirement to initiate uncoupled  $\text{Na}^+$ -transport is under investigation.

The experiment shown in Fig. 3 was performed in an incubation medium containing 40 mM  $\text{Na}^+$  and 50 mM  $\text{K}^+$ . ATP hydrolysis was monitored during net  $\text{Na}^+$   $\text{K}^+$  exchange (phase I) during uncoupled net  $\text{Na}^+$  transport (phase II) and at a steady state (phase III). In phase I 940 nmoles  $\text{Na}^+$  and 420 nmoles  $\text{K}^+$  were transported per mg protein for 400 nmoles ATP hydrolyzed.

From min 5 to min 25 (phase II) 540 nmoles  $\text{Na}^+$  were transported per mg protein and 550 nmoles ATP were hydrolyzed after subtraction of the ATP consumption due to passive backleak. Thus the coupling ratio was about  $1 \text{ Na}^+ : 1 \text{ ATP}$ . When compared to phase I one more  $\text{Na}^+$  has been transported and at least one more ATP has been hydrolyzed.

When the  $\text{Na}^+$ -gradient reached steady state ATP hydrolysis became linear with respect to time (phase III). This was expected since at steady state the limiting factor for ATP hydrolysis is passive backleak of  $\text{Na}^+$  and  $\text{K}^+$ .

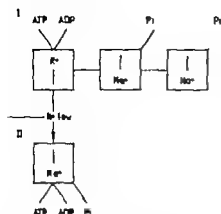


Fig. 4 Simplified scheme of the properties and functions of the purified Na<sup>+</sup>-K<sup>+</sup>-pump. The upper case contains the functional transport unit I which catalyzes coupled Na<sup>+</sup>-K<sup>+</sup>-transport with a stoichiometry of 2.2Na<sup>+</sup>/1K<sup>+</sup> (phase I). The dotted line designs the non-obligatory linkage. In the lower case the functional transport unit II which catalyzes active 'uncoupled' Na<sup>+</sup> transport (phase II) at coupling ratio of 1Na<sup>+</sup>/1ATP is presented. When the liposomes are depleted of K<sup>+</sup> after phase I the pump catalyzes transport of one more Na<sup>+</sup> but without K<sup>+</sup>-antiport.

## DISCUSSION

en Na<sup>+</sup>-K<sup>+</sup>-ATPase isolated from the renal outer medulla is reconstituted in phosphatidylcholine liposomes, a functional Na<sup>+</sup>-K<sup>+</sup> pump is obtained. In the present work the reconstituted Na<sup>+</sup>-K<sup>+</sup> transport system was used to study the nature of the Na<sup>+</sup>-K<sup>+</sup> coupling.

It was observed that the initial velocity of the Na<sup>+</sup>-K<sup>+</sup> exchange process increases when the Na<sup>+</sup> concentration in the incubation medium is increased at a constant K<sup>+</sup> concentration. At 50 mM Na<sup>+</sup> and 50 mM K<sup>+</sup> for instance the pump turns over 8 times more rapidly than at 20 mM Na<sup>+</sup> and 50 mM K<sup>+</sup>, yet the Na<sup>+</sup>-K<sup>+</sup> coupling ratio is invariably around 2.2Na<sup>+</sup>/K<sup>+</sup>. At high turnover rate the number of ATP molecules hydrolyzed appears to be equal to the number of K<sup>+</sup> transported. Accordingly the stoichiometry of the overall process is 2.2Na<sup>+</sup>/1K<sup>+</sup>/1ATP.

The observation that the Na<sup>+</sup>-K<sup>+</sup> coupling ratio is the same whether the pump turns over slowly at a low Na<sup>+</sup>-K<sup>+</sup> ratio in the incubation medium or more rapidly at a high Na<sup>+</sup>-K<sup>+</sup> ratio in the incubation medium may infer that the Na<sup>+</sup>-K<sup>+</sup> coupling is rigid. However, experiment on Na<sup>+</sup>-K<sup>+</sup>-ATPase

which has been selectively modified in its Na<sup>+</sup> form by trypsin treatment have revealed that 60% of the Na<sup>+</sup> transport capacity can be suppressed without affecting the K<sup>+</sup> transport capacity (Anner & Jørgensen 1979; Jørgensen & Anner 1979). Consequently countertransport of one Na<sup>+</sup> for one K<sup>+</sup> may be sufficient to sustain Na<sup>+</sup>-K<sup>+</sup> exchange. The dotted line in Fig. 4 symbolizes the link which is first exposed to trypsin in the presence of NaCl.

In the present report it has been emphasized that a fraction of the active Na<sup>+</sup> transport catalyzed by the Na<sup>+</sup>-K<sup>+</sup>-pump does not require counter-transport of K<sup>+</sup>. This 'uncoupled' net Na<sup>+</sup> transport starts when liposomes have been depleted at K<sup>+</sup>. The onset of uncoupled Na<sup>+</sup>-transport requires a minimal concentration of Na<sup>+</sup> in the incubation medium. 'Uncoupled' Na<sup>+</sup> transport discontinues when a maximal gradient of 3.3Na<sup>+</sup>/1K<sup>+</sup> is reached.

In conclusion the results reported herein infer that the purified Na<sup>+</sup>-K<sup>+</sup>-pump contains two functional transport units: one catalyzes coupled active Na<sup>+</sup>-K<sup>+</sup> transport and the second catalyzes 'uncoupled' active Na<sup>+</sup> transport. The simplified scheme shown in Fig. 4 illustrates how the transport units appear to be interacting: 'uncoupled' Na<sup>+</sup> transport starts when the pump has lowered the K<sup>+</sup> concentration at the K<sup>+</sup>-site to a limiting level.

A certain versatility of the Na<sup>+</sup>-K<sup>+</sup>-pump has been anticipated from studies in intact cells (Glynn & Karlish 1973). The present work shows that versatility is inherent in the purified Na<sup>+</sup>-K<sup>+</sup> pump and shows that the fundamental transport properties are preserved when the Na<sup>+</sup>-K<sup>+</sup> transport system has been isolated and reconstituted in artificial membranes. The protein part of the Na<sup>+</sup>-K<sup>+</sup>-pump is composed of two 100 000 mol wt catalytic subunits and of two to four 40 000-60 000 mol wt glycoproteins. It is reasonable to assume that the distinct transport patterns of the Na<sup>+</sup>-K<sup>+</sup> pump are the expression of specific configurations or interactions of the subunits. Therefore the reconstituted transport system will be a useful tool in the attempt to relate the physiological role of Na<sup>+</sup>-K<sup>+</sup>-ATPase with its molecular properties.

I wish to thank Mrs M. Moosmayer for excellent technical assistance.

## REFERENCES

- ANNER, B. M. & JØRGENSEN, P. L. 1979. Defective Na<sup>+</sup> transport by phospholipid vesicles reconstituted

- with selectively modified Na, K-ATPase. Proc 2nd Int Conf on the Properties and Functions of Na, K ATPase (ed J. C. Skou), in press. Academic Press, London and New York.
- ANNER, B. M., PITTS, B. J. R., LANE, L. K. & SCHWARTZ, A. 1976 Renal medulla Na, K ATPase reconstituted in vesicles coupled sodium and potassium transport. *The Pharmacologist* 18: 239.
- ANNER, B. M., LANE, L. K., SCHWARTZ, A. & PITTS, B. J. R. 1977 A reconstituted Na<sup>+</sup> K<sup>+</sup>-pump in liposomes containing purified (Na<sup>+</sup> + K<sup>+</sup>)-ATPase from kidney medulla. *Biochim Biophys Acta* 467: 340-345.
- DIBONA, D. R. & MILLS, J. W. 1979 Distribution of Na<sup>+</sup>-pump sites in transporting epithelia. *Fed Proc* 38: 134-143.
- GOLDIN, S. M. & TONG, S. W. 1974 Reconstitution of active transport catalyzed by the purified sodium and potassium ion-stimulated adenosine triphosphatase from canine renal medulla. *J Biol Chem* 249: 5907-5915.
- GLYNN, I. M. & KARLISH, S. J. B. 1975 The sodium pump. *Annu Rev Physiol* 37: 13-33.
- HILDEN, S. & HOKIN, L. E. 1975 Active potassium transport coupled to active sodium transport in vesicles reconstituted from purified sodium and potassium ion-activated adenosine triphosphatase from the rectal gland of *Squalus acanthias*. *J Biol Chem* 250: 465-496.
- HILDEN, S., RHEE, H. M. & HOKIN, L. E. 1974 Sodium transport by phospholipid vesicles containing purified sodium and potassium ion-activated adenosine triphosphatase. *J Biol Chem* 249: 7432-7440.
- JØRGENSEN, P. L. 1974 Isolation of (Na<sup>+</sup> + K<sup>+</sup>)-ATPase. *Methods Enzymol* 32: 277-290.
- JØRGENSEN, P. L. 1975 Isolation and characterization of the components of the sodium pump. *Quart Rev Biophys* 7: 239-274.
- JØRGENSEN, P. L. & ANNER, B. M. 1979 Purification and characterization of Na, K ATPase. VIII. Altered Na<sup>+</sup> K<sup>+</sup> transport ratio in vesicles reconstituted with purified Na, K-ATPase that has been selectively modified with trypsin in presence of NaCl. *Biochim Biophys Acta* 555: 485-492.
- KOEFOED-JØHNSEN, V. & USSING, H. H. 1958. The nature of the frog skin potential. *Acta Physiol Scand* 42: 296-308.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. R. 1951 Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265-275.
- RACKER, E. & FISHER, L. W. 1975 Reconstitution of an ATP-dependent pump with an ATPase from electric eel and pure phospholipids. *Biochem Biophys Res Comm* 67: 1144-1150.
- SCHWARTZ, A., NAGANO, K., NAKAO, M., LIN, DENMAYER, G. E., ALLEN, J. C. & MATSUI, H. 1971 The sodium and potassium-activated adenosine-triphosphatase system. *Methods Pharmacol* 1: 349.
- SCHWARTZ, A., LINDENMAYER, G. E. & ALLEN, J. C. 1975 The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol Rev* 27: 3-111.
- SKOU, J. C. 1957 The influence of some cations on adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta* 23: 394-401.
- SKOU, J. C. 1975 The (Na<sup>+</sup> + K<sup>+</sup>) activated enzyme system and its relationship to transport of sodium and potassium. *Quart Rev Biophys* 7: 401-434.





## Cholera toxin interactions with lipid bilayers

M. T. TOSTESON, D. C. TOSTESON and J. RUBNITZ

Department of Physiology, Harvard Medical School, Boston, Mass. 02115, USA

TOSTESON M. T., TOSTESON D. C. & RUBNITZ, J. Cholera toxin interactions with lipid bilayers. *Acta Physiol Scand* 1980 Suppl 481 21-25. Department of Physiology, Harvard Medical School, Boston, Mass. 02115, USA.

The purpose of the experiments described in this paper was to assess the binding of cholera toxin to bilayers containing its receptor the monosialoganglioside,  $G_{M1}$ . The assay was based on the fact that  $G_{M1}$  confers on the bilayer negative surface charge. The magnitude of this surface charge was estimated by measuring the electrical conductance ( $G$ ) of the bilayers exposed to monovalent  $K^+$  under conditions where  $G$  is directly proportional to the potassium concentration in the aqueous solutions immediately adjacent to the membrane surface. When bilayers were formed from mixtures of  $G_{M1}$  and glycerolmonooleate (GMO) it was found that the molar ratio of the lipids in the bilayer was the same as that in the membrane forming solution. It was further found that cholera toxin or the binding subunit of the toxin (cholera toxinogenoid) bind to GMO bilayers containing  $G_{M1}$  (but not to GMO bilayers containing phosphatidyl serine or dialkylganglioside  $G_{M2}$ ). The value of the apparent dissociation constant for the binding of cholera toxin to its receptor was found to be  $10^{-4}$  M comparable to values found in intact cells.

Work in many laboratories has shown that cholera toxin exerts its effect on cells through activation of the adenylate cyclase (as reviewed by Pierce et al 1971 and Frinkelstein 1976). Cholera toxin is a protein of approximately 84 000 daltons containing two different subunits A and B. The holotoxin of gomer is composed of one A (27 000-29 000 D) and 4-6 B (10 000-12 000 D) subunits. The A subunit consists of two peptide chains,  $A_1$  (20 000-5 000 D) and  $A_2$  (2 500-9 700 D) linked through a disulfide bond (Klapper et al 1976). The activation of the adenylate cyclase by cholera toxin in intact cells begins with the binding of the toxin to the cell surface receptor, the monosialoganglioside  $G_{M1}$ . The specific interaction of cholera toxin with the receptors is mediated through the B subunits of the protein (cholera toxinogenoid) (Cuatrecasas 1973, van Heyningen 1974). The subsequent events which lead to the time-dependent activation of the cyclase are less well understood. It has been proposed that this lag period between exposure and cyclase activation which varies from 30 to 120 min depending on the type of cell under study, may represent the time required for lateral motion of the toxin on the cell

surface (Bennett et al 1975), penetration of the biologically active A peptide into or through the plasma membrane (Gill & King 1975) and the covalent modification of a cellular protein in an NAD-dependent ADP-ribosylation reaction catalyzed by the A peptide (Gill 1975). The activation of the cyclase by cholera toxin would then proceed through this ADP-ribosylation of an as yet unidentified intracellular protein.

Our interest in cholera toxin was stirred by a report of Moss et al (1977) in which they showed that cholera toxin interacts with lipid vesicles promoting an increase in their glucose permeability. We decided to study the possibility that the toxin-receptor interaction might lead to changes in the ionic permeability of membranes anteceding its effect on adenylate cyclase. We have shown (Tosteson &

Abbreviations used: GMO = glycerolmonooleate; PS = phosphatidyl serine; PC = phosphatidyl choline;  $G_{M1}$  = galactosyl-N-acetyl-galactosaminyl-(N-acetylneuraminyl)-galactosyl-glucosyl-ceramide;  $G_{M2}$  = N-acetylneuraminyl-galactosyl-(N-acetyl-galactosaminyl)-(N-acetylneuraminyl)-galactosyl-glucosyl-ceramide.

Tosteson 1978) that when cholera toxin was added to one of the aqueous solutions surrounding a planar bilayer made with glycerolmonooleate and  $G_{M}$  it interacts with the bilayer promoting an increase in membrane conductance. The magnitude of this increase was found to depend on the cholera concentration in the aqueous solutions. Using different concentrations of  $G_{M}$  in the membrane forming solution, we have further shown that the toxin concentration required to produce half the maximal steady-state conductance increase  $1.2 \times 10^{-7} M$  is very similar to the value found by Cuatrecasas (1973) for the interaction of cholera toxin with fat cell membranes. Finally we have also shown that the increase in membrane conductance is associated with the formation of channels, with a unit conductance of 20–40 pmho.

In this paper we describe experiments designed to measure the binding of holotoxin and cholera toxinogen to GMO bilayers containing 10%  $G_{M}$ . The results of these experiments show that both cholera holotoxin and cholera toxinogen bind to these bilayers and that the apparent dissociation constant for the process is  $10^{-7} M$ .

## MATERIALS AND METHODS

### (a) Membrane formation and electrical measurements

Bilayers were formed from GMO or from a mixture of GMO and  $G_{M}$  in the molar ratios indicated, by apposition of two monolayers spread at the air-solution interface (Moelster & Mueller 1977). The aqueous solutions present in the 1.5 ml chambers were buffered to pH 7.0 with 1 or 5 mM TrisCl. The contents of both chambers were stirred continuously with magnetic stirrers. The steady-state membrane conductance was determined in the limit of zero applied potential by measuring the steady-state current flowing across the membrane in response to an applied potential difference using silver-silver chloride electrodes.

### (b) Surface charge measurement

The surface charge density of GMO bilayers containing monodialogangliosides was determined using nonactin-K as a probe for surface potentials. Bilayers were made from GMO +  $G_{M}$  in the presence of 1 mM KCl and  $6.6 \times 10^{-6}$ – $10^{-7} M$  nonactin buffered to pH 7.0 with 1 mM TrisCl. After the membrane conductance ( $G$ ) had reached steady value the ionic strength of the aqueous solutions was increased by additions of aliquot of a concentrated solution of LiCl, a non-permeant cation, and  $G$  was determined after each addition. The surface charge density  $\sigma_s$  was then obtained by fitting the experimental point to the equation (McLaughlin et al. 1970)

$$G = A \left[ \frac{136\sigma}{\sqrt{\Sigma c_i}} + \sqrt{\left( \frac{136\sigma}{\sqrt{\Sigma c_i}} \right)^2 + 1} \right]^2 \quad (1)$$

where  $A$  is a constant (at constant  $K$  and nonactin concentrations) equal to the membrane conductance at infinite salt concentration, and  $c_i$  is the molar concentration of all ionic species.

### (c) Binding of cholera toxin and cholera toxinogen

Binding of holotoxin or cholera toxinogen was determined through measurements of the change in the surface charge of the bilayers. Bilayers containing monodialoganglioside were formed in the presence of 1 mM KCl, 29 mM LiCl and  $10^{-7} M$  nonactin buffered to pH 7.0 with 5 mM TrisCl. After determination of the steady-state membrane conductance aliquots of cholera toxin (or cholera toxinogen) were added to both sides of the membrane and the conductance  $G_p$ , determined after each addition. The number of molecules of  $G_{M}$  bound ( $\sigma_b$ ) were then calculated according to the following equations Hladky & Haydon (1973):

$$\ln G_p/G_s = -\frac{F}{RT} \Delta\phi_p \quad (2)$$

and

$$\sigma = \sigma_s - \frac{\sqrt{C}}{136} \sinh \frac{(\Delta\phi_s - \Delta\phi_p)F}{2RT} \quad (3)$$

where  $G_s$  is the membrane conductance prior to the addition of proteins,  $\sigma$  is the surface charge density obtained through equation 1 and  $\Delta\phi_p$  is the interfacial potential due to this surface charge density at a total electrolyte concentration.

### (d) Materials

Glycerolmonooleate was purchased from Nu-Chek, PS and the mono- and disialogangliosides were obtained from Sepelco Inc. and cholera toxin from Schwarz Mann. Cholera toxinogen was a gift from Dr R. A. Finkelstein (Dept. of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas).

## RESULTS AND DISCUSSION

### (a) Membrane composition

Fig. 1 shows the results of experiments designed to estimate the surface density of  $G_{M}$  molecules in the bilayers. The fit of the experimental points to equation 1 (Methods) yields a value of 10 charges/Å for pure GMO membranes and  $8.3 \times 10^{-4}$  and  $25 \times 10^{-4}$  charges/Å for bilayers made from solutions containing  $G_{M}$ , GMO molar ratios of 0.03 and 0.1 respectively. Since there is one charge per molecule of  $G_{M}$  and the area per molecule of GMO is 40 Å

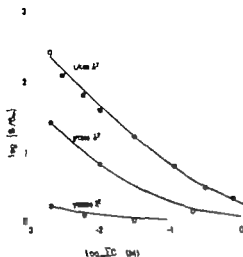


Fig. 1 Determination of bilayer composition. Bilayers were formed from pure GMO (curve labelled  $1/10000 \text{ \AA}^2$ ) or from GMO containing  $G_{M2}$  at 0.03 (curve labelled  $1/100 \text{ \AA}^2$ ) or 0.1 (curve labelled  $1/400 \text{ \AA}^2$ ) molar ratio in the presence of  $1 \text{ mM KCl} + 1 \text{ mM Tris-Cl}$  pH 7.0,  $6.6 \times 10^{-6} \text{ M}$  nonactin. The ionic strength was increased by addition of concentrated LiCl containing the same concentration of the other ions and nonactin as in the initial solution. Points are mean values obtained in 4 or more different membranes. The lines were drawn according to Equation 1 (Methods) with the value of  $\sigma$  as indicated for each line.

(Hladky & Haydon 1973) these charge densities correspond to 0.03 and 0.1 molecules of  $G_{M2}$  per molecule of GMO values which coincide with the composition of the lipid mixtures used to form the bilayers.

These results extend those of HBI & Lester (1972) who found that when gangliosides were rehydrated together with phosphatidylcholine the ratio of ganglioside to PC molecules in lamellae of a combined ganglioside-lecithin smectic mesophase was of the same composition as the initial mixture (up to molar ratios of 0.3).

(b) Binding of cholera toxin and choleraenoid

Fig. 2 shows the effect of cholera toxin and choleraenoid on the nonactin-induced  $K^+$  conductance of GMO bilayers containing 10%  $G_{M2}$ . The values on the ordinate are the ratios of the conductance in the presence to that in the absence of toxin (or choleraenoid). The results depicted in the figure show that addition of either of the proteins lowers the mem-

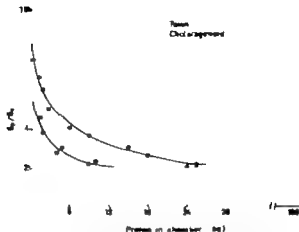


Fig. 2 Effect of choleraenoid (O) and cholera toxin (●) on the nonactin- $K^+$  induced conductance.  $G_{M2}$ +GMO 0.1 molar ratio bilayers were formed in  $1 \text{ mM KCl} + 29 \text{ mM LiCl} + 5 \text{ mM Tris-Cl}$  (pH 7.0) in the presence of  $10^{-6} \text{ M}$  nonactin. Stock protein solutions (choleraenoid:  $5 \text{ mg/ml}$ ; cholera toxin:  $0.5 \text{ mg/ml}$ ) were diluted prior to use with the initial medium and small portions were added to both aqueous phases bathing the bilayer. Volume of chambers:  $1.5 \text{ ml}$ . Points represent mean values obtained in at least 4 different membranes.

brane conductance to 20% of the value in the absence of protein. This suggests that the interaction of these proteins with the ganglioside molecules in the bilayer lowers the surface charge density of the bilayer by about 80%. This estimate of the fraction of  $G_{M2}$  charges neutralized by cholera toxin (or choleraenoid) was confirmed by the results of the experiments described in Fig. 3. The residual surface charges remaining after addition of high concentrations of proteins was estimated by increasing the ionic strength of the bathing solutions. The value for the surface charge density obtained  $8.3 \times 10^{-4} \text{ charges/\AA}^2$  indicates that after exposure to toxin (or choleraenoid) the surface charge density of  $G_{M2} + \text{GMO}$  bilayers is 20–30% of the initial value.

The specificity of the toxin binding to its receptor was tested by changing the lipid mixture of the bilayers. It was found that addition of toxin (or choleraenoid) to GMO bilayers containing phosphatidyl serine (0.1 molar ratio) or disialoganglioside  $G_{D2a}$  (molar ratio 0.08) did not change the initial nonactin induced conductance of these bilayers. Changing the ionic strength of the solutions bathing these bilayers after addition of high



## Ion movement through gramicidin A channels

On the importance of the aqueous diffusion resistance and ion-water interactions

OLAF SPARRE ANDERSEN and JOAQUIM PROCOPIO

Department of Physiology and Biophysics, Cornell University Medical College, 1300 York Avenue, New York, New York 10021 USA

ANDERSEN O S & PROCOPIO J. Ion movement through gramicidin A channels. On the importance of the aqueous diffusion resistance and ion-water interactions. Acta Physiol Scand 1980 Suppl. 481 27-35. Department of Physiology and Biophysics, Cornell University Medical College, 1300 York Avenue, New York, NY 10021 USA.

The movement of an ion through a membrane channel proceeds in at least five separate steps. Diffusion through the aqueous phases up to the channel, association with the channel itself, translocation through the channel, dissociation from the channel, and diffusion through the aqueous phases out from the channel. We demonstrate that, contrary to current working assumptions, the aqueous diffusion step may be an important determinant of overall ion movement through the channel. We further describe the kinetics of Na<sup>+</sup> movement through gramicidin A channels. Using these data we show that one will have to consider the movement of H<sub>2</sub>O through the channel explicitly in any complete model for ion translocation through the channel interior.

The molecular mechanisms by which integral membrane proteins permit specific solute permeation through the membrane are not known in detail. But it is likely that the translocation of small inorganic ions—and probably most other polar solutes—proceeds through a pre-formed path lined with polar groups which spans part or all of the hydrocarbon core of the membrane. If this structure forms a continuous pathway from one aqueous phase to the other, one may call it a channel.

The permeability properties of a channel will, among other factors, depend upon its dimensions. In one extreme case the channel can be looked upon as a large water-filled structure through which ion movement proceeds essentially as a free electrodiffusion process. The selectivity among ions of the same valency will largely be determined by the aqueous mobility of the ions. Selectivity among ions of different valency will, in addition, be influenced by electrostatic factors. In the other extreme case the channel is a quite narrow structure, an array of coordination sites which allow the movement of ions and water to occur as a series of rapid jumps from one potential energy minimum to an adjacent—with pauses for various times at each site

(potential energy minimum). The movement of ions and water proceeds in an orderly fashion as neither ions, H<sub>2</sub>O molecules, nor ions and H<sub>2</sub>O molecules can overtake each other: the channel exhibits *single-file* behavior (Hodgkin & Keynes 1955; Fleckmann 1965a; Hille & Schwarz 1978; Finkelstein & Rosenberg 1979). In biological membranes an example of the first channel type is the acetylcholine-activated channel in the neuromuscular junction (Dwyer et al. 1979). An example of the second channel type is the delayed rectifier (K<sup>+</sup>) channel in nerve and muscle (Hodgkin & Keynes 1955; Armstrong 1975; Hille & Schwarz 1978; Benigisch 1979).

The fact that one can divide channels in biological membranes into several different categories, does not imply that one understands the molecular mechanisms involved in ion translocation or ion

---

It is not known whether ions move by a single-file mechanism relative to water in the K<sup>+</sup> channel. The dimensions of the K<sup>+</sup> channel (Hille 1975b) make this likely possibility. The electro-osmotic potentials observed by Vargas (1968) may indeed reflect ion/water single-fileing.

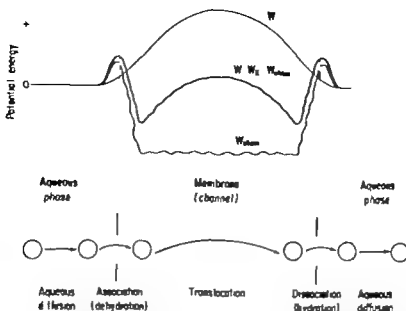


Fig. 1 Schematic representation of a model for ion translocation through the Gram A channel. Top. The potential energy profile.  $W_{chan}$  denotes the qualitative variation in the local interactions between the ion and the wall.  $W_e$  denotes the variation in the electrostatic image energy as an ion moves through the channel (Levitt 1978). The potential energy profile  $W$  is the sum of  $W_e + W_{chan}$ . Bottom. A schematic illustration of the five separate steps involved in the movement of an ion from one aqueous phase to the other.

specificity in detail. Progress towards this goal will probably be slow in view of the unknown and probably quite complicated structure of these channels. Studies on channel-mediated permeability phenomena in artificial lipid bilayers are therefore likely to contribute significantly to a deeper understanding of ion movement through channels in biological membranes. Examples of wide water-filled channels are the polyene-channels (Finkelstein & Holz 1973; Andreoli 1974). An example of a narrow channel exhibiting single file properties is the gramicidin A channel (Levitt et al. 1978; Rosenberg & Finkelstein 1978a; Schagina et al. 1978; Procopio & Andersen 1979).

## GRAMICIDIN A

Gramicidin A (gram A) forms ion-selective channels which span lipid bilayer membranes (Hladky & Haydon 1972). The chemical structure of the channel is known (Sarges & Witkopf 1965) and considerable evidence is available concerning the conformation of the channel (Urry et al. 1975; Bamberg et al. 1977; Veatch & Stryer 1977; Koeppe et al. 1978). Gram A channels are cation selective (Myers

& Haydon 1977; Urban et al. 1978; but see Eisenman et al. 1977) and exhibits a respectable selectivity among monovalent cations (Myers & Haydon 1972; Eisenman et al. 1978; Urban et al. 1978). Gram A channels further exhibit *mole-fraction-dependent conductance changes* (Andersen 1975; Neher 1975; Eisenman et al. 1977; Andersen 1978; McBride & Szabo 1978; Neher et al. 1978). Gram A channels thus exhibit permeability properties which are phenomenologically similar to what can be observed in channels in excitable membranes (Hagiwara & Takahashi 1974; Cahalan & Begenisich 1976). There are a number of important differences between the Gram A channel and channels in biological membranes, most notably the absence of fixed charges in the channel itself. Studies of the permeability properties of the very simple gram A channel should nevertheless be able to contribute significantly to the understanding of channel-mediated permeability phenomena in biological membranes.

## PERMEABILITY

Ion fluxes through channels have traditionally been described in terms of an electrodiffusion formalism.

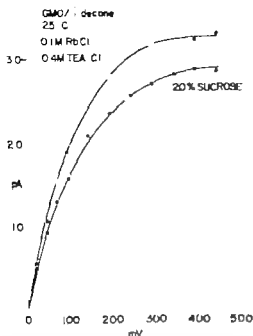


Fig. 2. Current-voltage characteristics of Gram A single channels in glycerolmonooleate/n-decane membranes. The aqueous phases contain symmetrical 0.1 M RbCl + 0.4 M tetraethylammonium chloride with (O) or without (●) 20% (w/v) sucrose. The points are the mean values of between 115 and 1037 current transitions at different potentials. The standard deviations are always less than 14% of the mean values in all cases. The conductivity of the aqueous phases decreases by 28% upon the addition of sucrose.  $T = 23^\circ\text{C}$ .

either the integrated version of the Nernst Planck flux equations as obtained by Goldman (1944) and Hodgkin & Katz (1949) or the equivalent circuit formalism of Hodgkin & Huxley (1952) Finkelstein & Mauro (1963) should be consulted for an incisive analysis of both formalisms. Both the above approaches assume free movement of ions and water relative to one another which may be adequate to describe ion movement through wide channels. But such formalisms are unable to describe transport mechanisms that exhibit more complicated behavior such as saturation of the flux, competition among several permeant or impermeant ions, concentration-dependent ion selectivity and coupling among the fluxes of several species (Hille 1978).

Alternative models for ion translocation through narrow channels have therefore been constructed on the basis of a finite one-dimensional random walk model with transition probabilities which may depend upon position, applied potential and the

number (and type) of ions which may occupy the channel at any time (Hodgkin & Keynes 1955; Heckmann 1965a, b; Lüscher 1973; Markin & Chizmadzev 1974; Hille 1975a, b; Aytan et al. 1977; Sandblom et al. 1977; Hille & Schwarz 1978; Levitt 1978b; Häggblom et al. 1979; Urban & Hladky 1979). The essential features of such models are illustrated in Fig. 1. The potential energy profile possesses a minimum near each of the channel openings; the channel possesses two sites<sup>1</sup> in the single-file region—but it may have additional binding sites exterior to the channel proper. The two potential energy minima are separated from each other by potential energy barriers. The movement of an ion over each of the barriers can be represented by a series of discrete jumps which can be described by the absolute rate theory (Parlin & Eyring 1954). Alternatively one may regard each of the steps to occur as a single jump—in which case the strict applicability of the absolute rate theory becomes problematical. In both situations one can make the rate of crossing the various barriers to depend upon the number of ions occupying the channel. At present neither formalism considers explicitly the movement of any  $\text{H}_2\text{O}$  molecules which occupy the channel—and may constrain the ion movement.

The present models have been developed using a number of simplifying assumptions, some of which may be incorrect. Firstly it is generally assumed that any limitations imposed by the aqueous diffusion step can be disregarded. Secondly it is usually assumed that the ions move by a *vacancy* mechanism in which the ions only can move into a site that has been vacated by its previous occupant. This may not be a reasonable assumption in a channel that only contains a few ions (and  $\text{H}_2\text{O}$  molecules). It is possible that the ions also can make use of the *knock-on* mechanism in which the ions move by "colliding" with each other thus causing the displacement. This mechanism could become particularly pronounced when a potential difference is applied across the membrane. The resulting flux equations will differ appreciably at high occupancies of the channel (Hodgkin & Keynes 1955; Heckmann & Vollmerhaus 1970). Thirdly in channels where ions and water are constrained to move in single-file, do the ions and water relative

<sup>1</sup> This usage of the word *sites* differs that introduced earlier.



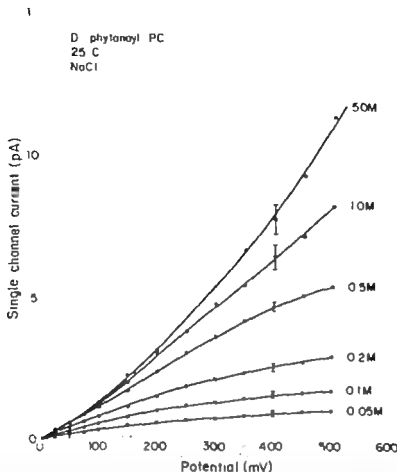


Fig. 3 Current-voltage characteristics of gram A single channels in diphytanoyl/phosphatidylcholine/ $\alpha$ -decane membranes. The aqueous phases contained symmetrical NaCl at the concentrations indicated. The points are mean values of 68 to 2261 (usually 700–800) current transitions of each potential. The vertical bars at 400 mV indicate the standard deviations at these potentials. The standard deviation is always less than 20% (usually less than 10%) of the mean value.  $T=25^{\circ}\text{C}$ .

to each other move by a *vacancy* or a *knock-on* mechanism—and do they stay in the same mode at all applied potentials and ion occupancies? If the ions relative to  $\text{H}_2\text{O}$  move in the *vacancy* mode then it should be reflected in a very weak voltage-dependence of the rate constant for the individual steps. It further becomes necessary to consider the role of any  $\text{H}_2\text{O}$  molecule which separate two ions when deciding whether to use the *vacancy* or the *knock-on* mode to describe the ion movement in such a multiply occupied channel.

We presently demonstrate that contrary to current working assumptions the aqueous diffusion step may be an important determinant of the observed kinetics of ion movement through the membrane and that one probably will have to consider

the  $\text{H}_2\text{O}$  movement through the channel explicitly in any successful model for ion translocation through the gram A channel.

### AQUEOUS ACCESS LIMITATION

The shape of the current-voltage characteristics of gram A single channels depends upon the aqueous concentration of the permeant ion (Hladky & Haydon 1972; Håglund et al. 1979) see also Fig. 3. At low concentrations the single channel current reaches a limiting value  $I_{\infty}$  with increasing potential as illustrated in Fig. 3. This indicates that the ion movement is limited by a voltage-independent step which can be unmasked at permeant ion concentrations sufficiently low that the channel is far

from saturation. Such limiting currents are most easily demonstrated using very permeant ions such as Rb, but they can also be inferred for less permeant ions such as Na—see Fig. 3. The magnitude of  $I_{\text{lim}}$  is about 3.2 pA in 0.1 M RbCl + 0.4 M TEA Cl. This value is consistent with a diffusion-controlled movement of Rb<sup>+</sup> up to the channel—which is represented as a hemi-spherical sink in a plane interface with a capture radius<sup>2</sup> of approximately 0.05  $\mu\text{m}$  (Lüger 1976). The theoretically calculated value for  $I_{\text{lim}}$  is about 5 pA, in reasonable agreement with the measured value of about 3.2 pA—considering the approximations made in the calculations.

If the magnitude of  $I_{\text{lim}}$  is determined by diffusion through the aqueous phases up to the channel, one would predict that an increase in the aqueous viscosity would cause a decrease in the magnitude of  $I_{\text{lim}}$ . This is indeed the case, as illustrated in Fig. 2. When the aqueous phases contain 20% sucrose which almost doubles the viscosity,  $I_{\text{lim}}$  decreases to about 2.8 pA. The relative decrease in  $I_{\text{lim}}$  is 45% of the decrease in aqueous solution conductivity. This discrepancy is expected, as the major diffusion resistance occurs with 0.1–0.2  $\mu\text{m}$  of the channel (Andersen 1980), a distance much smaller than the dimensions of the sucrose molecule.

From the magnitude of  $I_{\text{lim}}$ , one can calculate the magnitude of the convergence permeability up to the channel,  $3.4 \times 10^{-14}$  l/cm<sup>2</sup>(channel  $\times$  sec) for Rb. The convergence conductance of each of the aqueous phases is therefore 125 pS (Lüger 1976). This value should be compared with the small-signal single channel conductance, 22 pS. Only 2/3 of the measured single channel resistance originates in the channel itself. Increasing the viscosity of the aqueous phases will therefore be expected to decrease the single channel conductance at all potentials as observed in Fig. 2. At low potentials the observed decrease is however larger than predicted.

Experiments with the alkali metal ions and NH<sub>4</sub><sup>+</sup> show a uniform pattern—the magnitude of the single channel currents at high potentials (450–550 mV) is consistent with a diffusion-limited ion entry into the channel (Andersen 1978; Andersen & Procopio 1979b). Two ions stand out from this pattern: Ag<sup>+</sup> and Tl<sup>+</sup>. The currents at 500 mV do not show definite signs of reaching their limiting value, and are more than two times larger than predicted from the alkali metal ion experiments (Andersen 1978; An-

dersen & Procopio 1979b). This finding implies that the effective capture radius of the gram A channel has increased—by 100% or more. Such an increase could be caused either by an increase in the channel diameter per se—as a result of a structural rearrangement, or by the existence of additional binding sites exterior to the channel proper. The ions could move from these binding sites into the channel by a mechanism resembling lateral diffusion. The general features of such a mechanism have been analyzed by Adam & Delbrück (1967) who found that it can increase the effective capture radius several fold.

One can distinguish between the two possible mechanisms by which the effective capture radius is increased by electrical measurements (Andersen 1980) or by using small non-electrolytes as probes of the channel dimensions. The interpretation of changes in permeability coefficients for a permeable solute is however very difficult in a single-file channel, as the permeability will be affected by changes in channel dimensions as well as by blocking of the channel by ion bindings as described by Dani & Levitt (1979). But the gram A channel is normally impermeable to urea (Finkelstein 1974) while it is permeable to the only slightly smaller foramide (Procopio & Andersen, unpublished observations). If the increase in effective capture radius reflects an increase in the luminal diameter of the channel, this should be registered as a measurable urea flux through the channel. This was not observed, see Table 1. We find that the unmodified membrane permeability for urea is unaffected by the composition of the aqueous phases, and that there is no detectable urea flux through the gram A modified membranes, irrespective of the cation present. We conclude that Ag<sup>+</sup> and Tl<sup>+</sup> do not produce major changes in channel structure. A similar result has been obtained from studies of the single channel currents of various mole fractions of Ag<sup>+</sup> or Tl<sup>+</sup> and Cs<sup>+</sup> (Andersen 1980). These investiga-

<sup>2</sup> The capture radius,  $a_c$ , is the difference between the channel radius,  $R$ , and the ion radius,  $r$  (Ferry 1936; Lüger 1976). The relevant magnitude of each of these will be determined by the detailed mechanism through which the ion reaches the channel mouth—especially how the hydration water is handled. A likely maximum estimate for  $a_c$  can be obtained as the difference between the luminal radius of the channel, about 0.2  $\mu\text{m}$  (Urry et al. 1975) and the crystal radius of Rb<sup>+</sup>, 0.15  $\mu\text{m}$  (Häde 1973b).

Similar results have been obtained by Tredgold & Jones (1979) who changed the viscosity of the aqueous phases by substituting the more viscous D<sub>2</sub>O for H<sub>2</sub>O.

Table 1 Urea permeability of lipid bilayers

Mean  $\pm$  S.D. The number in parenthesis indicates the number of experiments. Di-phytanoylphosphatidylcholine/n-decane membranes. 25°C. All solutions contained 10 mM urea. The conductance of the gramicidin A modified membranes was larger than 1 S/cm<sup>2</sup>.

Salt	Without gramicidin A (cm/sec)	With gramicidin A (cm/sec)
NaCl 1.0 M	$6.0 \pm 0.7 \times 10^{-7}$ (4)	$5.2 \times 10^{-7}$ (4)
AgNO <sub>3</sub> 1.0 M	$6.2 \times 10^{-7}$ (3)	$6 \times 10^{-7}$ (1)
TiNO <sub>3</sub> 0.3 M	$8.4 \times 10^{-7}$ (2)	$6.1 \times 10^{-7}$ (2)

tions have additionally provided more direct evidence for the binding of Ag<sup>+</sup> and Ti<sup>3+</sup> to some outer binding sites.

There is, however, some evidence that the molecular behavior of the gram A channel may be altered by Ag<sup>+</sup> and Ti<sup>3+</sup> relative to what is observed with the alkali metal ions and NH<sub>4</sub><sup>+</sup>. The average channel lifetime is about 5 times longer in the presence of Ag<sup>+</sup> and Ti<sup>3+</sup> than in the control situation (Andersen & Procopio 1979b). It is presently not known whether the intrinsic permeability properties of the channel are specifically affected by Ag<sup>+</sup> or Ti<sup>3+</sup>—apart from the effects brought about by multiple ion occupancy and occupancy-dependent rate constants for moving across the various barriers.

## ION MOVEMENT THROUGH THE CHANNEL

The details of the kinetics of ion movement through conducting channels can be obtained by analyzing the single channel current-voltage characteristics over a range of aqueous salt concentrations and as large potential variation as technically possible. Fig. 3 illustrates such data, obtained with Na<sup>+</sup> as the current carrier. It should be noted that (a) at low salt concentrations the current tends to reach a limiting value with increasing potential—reflecting the importance of the aqueous diffusion step and (b) at high salt concentrations the currents increase “exponentially” with increasing potential—reflecting the permeability properties of the channel itself.

A kinetic analysis of these data is greatly facilitated by the observation that the movement of

<sup>23</sup>Na<sup>+</sup> through the channel can be described by the Ussing (1949) flux-ratio equation (Procopio & Andersen 1979). This may indicate that there can be no more than one Na<sup>+</sup> in the channel at attainable salt concentrations although it is possible that the channel could be occupied by two Na<sup>+</sup>. If the major barrier for ion movement through the channel is the translocation step across the central barrier in Fig. 1 (Heckman 1965b; Hille & Schwarz 1978; Urban & Hladky 1979).

Details of the kinetic analysis and of the control experiments will be given elsewhere. The results indicate that it is the former possibility (only one ion) which is correct—although small systematic discrepancies between the predictions of simple model and the observed data occur at very high salt concentrations (Andersen & Procopio 1979a). We believe that these discrepancies reflect the variation in water activity which occurs at very high salt concentrations. Table 2 summarizes our results for gram A in bacterial phosphatidyl-ethanolamine/n-decane membranes. We also list the water permeability obtained from Rosenberg & Finkelstein (1978b). It should be noted that the rate constant for translocation through the channel interior is larger than the rate constant for exit out of the channel. Ion movement through the channel is limited by the exit step contrary to intuition which would locate the major barrier in the channel interior. A qualitatively similar result has been found for gram A channels in glycerolmonooleate/n-decane and diphytanoylphosphatidylcholine/n-decane membranes. The position of the potential energy minima appears to be very similar for all three lipids, about 65% of the applied potentials falls across the central barrier.

It is known that Na<sup>+</sup> and H<sub>2</sub>O cannot pass each other in the gram A channel (Levitt et al. 1978; Rosenberg & Finkelstein 1978a). But it is not known whether the Na<sup>+</sup> movement is limited by the movement of Na<sup>+</sup> *per se* or whether it is limited by H<sub>2</sub>O movement. It is therefore of interest to compare the rate constant for Na<sup>+</sup> translocation through the channel interior to the rate of H<sub>2</sub>O translocation which can be estimated to be about

— The chemical identity of these outer sites is presently unknown. They are part of the gram A channel itself as their existence has been inferred in measurements made on channels incorporated into very different membranes, e.g. glycerolmonooleate/n-decane and diphytanoylphosphatidylcholine/n-decane membranes.

Table 2. *Kinetics of Na<sup>+</sup> movement through gramicidin A channels*

Bacterial phosphatidylethanolamine/n-decane membranes, 23±1°C.	
Convergence permeability	~10 <sup>-22</sup> liter/sec
Maximal conductance	14.6 pS
Activity for half-maximal conductance	0.23 m
Dissociation constant for a single site	0.30 m
Rate constant for the dissociation step	1.2×10 <sup>10</sup> ions/(channel×sec)
Rate constant for the association step	7×10 <sup>-27</sup> liter/(channel×sec)
Rate constants for the translocation step	×10 <sup>10</sup> ions/(channel×sec)
Diffusional water permeability*	×10 <sup>-22</sup> cm <sup>2</sup> /sec

\* From Rosenberg & Finkelstein (1978b).

$6.7 \times 10^7$  H<sub>2</sub>O molecules/(channel×sec) using the permeability coefficient listed in Table 2. The gram. A channel contains approximately 5–6 H<sub>2</sub>O (Rosenberg & Finkelstein 1978a, b). If one assumes that the electrical distance separating the two potential energy minima approximates the real distance then we find that 3–4 H<sub>2</sub>O molecules separate the two potential energy minima. We thus predict the rate constant for Na<sup>+</sup> translocation through the channel interior to be about  $2 \times 10^7$  ions/(channel×sec)—which is the independently determined value. It is therefore very likely that the Na<sup>+</sup> movement is water-limited, but we do not know whether ions and H<sub>2</sub>O move by the vacancy or knock-on mode at low potentials.

This correlation indicates, nevertheless, that the magnitude of the central potential energy barrier  $W$  may be considerably smaller than indicated in Fig. 1.

At high potentials it appears that ions can push H<sub>2</sub>O ahead of itself out of the channel. At 500 mV and 5.0 M NaCl the single channel current, in bacterial phosphatidylethanolamine/n-decane membranes, is about 11 pA and shows no sign of reaching a limiting value. This current corresponds to a movement of  $7 \times 10^7$  ions/(channel×sec) which corresponds to about  $4 \times 10^8$  H<sub>2</sub>O molecules/(channel×sec), or 6 times the unidirectional H<sub>2</sub>O flux at zero potential. A simple argument based upon the concept of first passage times (Feller 1967) shows

that the largest water flow which can be associated with ion movement through a single-file channel operating in the vacancy mode is about 3 times the unidirectional water flux at equilibrium measured by tracer  $7 \times 10^7$  H<sub>2</sub>O/(channel×sec) (Andersen manuscript in preparation). The observed Na<sup>+</sup> movement is thus about two times larger than the upper limit for a single-file channel in the vacancy mode. The discrepancy is significant, but not glaringly so. It is therefore important to note that currents greater than 20 pA have been observed with NH<sub>4</sub><sup>+</sup> as the current carrier. At high potentials ions can indeed knock H<sub>2</sub>O molecules out of the channel. In retrospect, this conclusion is not surprising as an ion gains a potential energy of about 20 kT if the potential is 500 mV.  $k$  is Boltzmann's constant,  $T$  is temperature in K. The present data still leave open the question of whether ions and H<sub>2</sub>O at low potentials move in the vacancy or knock-on mode, as well as the question of ion-ion interactions.

We wish to thank Cheryl K. Martin and Frank I. Navetta for excellent technical assistance and Rochelle Lester for secretarial assistance.

This work was supported by N. L. H. Grant no. GM 21342. Olaf S. Andersen was supported by a Senior Investigatorship from the New York Heart Association. Joaquim Procópio was supported by a fellowship from F.A.P.E.S.P. São Paulo, Brazil.

## REFERENCES

- ADAM G. & DELBRÜCK, M. 1968. Reduction of dimensionality in biological diffusion processes. In: *Structural chemical and molecular biology* (ed. N. Davidson and A. Rich) pp. 198–215. W. H. Freeman, San Francisco.
- AIYTAN S. R., KALANDADZE, I. L. & CRUZ MADIEV Y. A. 1977. Ion transport through the potassium channels of biological membranes. *Bioelectrochem Bioenerg.* 4, 30–44.
- ANDERSEN O. S. 1975. Ion-specificity of gramicidin A channels. 5th International Biophysics Congress, p. 112 (Abstract).
- ANDERSEN O. S. 1978. Ion transport across simple membranes. In: *Renal function* (ed. G. H. Oelbleich and E. Purcell), pp. 71–99. Independent Publishers Group, Port Washington, N. Y.
- ANDERSEN O. S. 1980. Kinetics of ion movement through gramicidin A channels. I. The aqueous diffusion step. *Biophys. J.* (in preparation).

Much larger currents have of course been observed using other lipids, but it is not known whether the H<sub>2</sub>O permeability coefficient is constant irrespective of the type of lipid into which the channel is incorporated.

ANDERSEN O S & PROCOPIO J 1979a The kinetics of Na<sup>+</sup> movement through gramicidin A channels. *Biophys J* 25 8a.

ANDERSEN O S & PROCOPIO J 1979b Ion entry into gramicidin A channels is diffusion-limited. *Bull New York Acad Med* (in press)

ANDREOLI T E. 1974 The structure and function of amphotericin B-cholesterol pores in lipid bilayer membranes. *Ann N Y Acad Sci* 235 448-468.

ARMSTRONG C. M. 1975 Potassium pores of nerve and muscle membranes. In: *Membranes. Vol. 3 Lipid bilayers and biological membranes: dynamic properties* (ed. G. Eisenman) pp. 345-358 M. Dekker New York.

BAMBERG E., APELL H J & ALPES H. 1977 Structure of the gramicidin A channel. Discrimination between the  $\alpha$  and  $\beta$  helix by electrical measurements with lipid bilayer membranes. *Proc Natl Acad Sci* 74 (6): 403-2406.

BEGENISICH T 1979 Ionic interactions in potassium channels. In: *Membrane transport processes Vol 3* (ed. C. F. Stevens and R. W. Telen), pp. 105-111 Raven Press, New York.

CAHALAN M & BEGENISICH T 1976 Sodium channel selectivity: dependence of internal permanent ion concentration. *J Gen Physiol* 68 111-135

DANI J A. & LEVITT D G 1979 Partitioning of cations into the gramicidin channel determined from cation blockage of water flux. *Biophys J* 35 66a.

DWYER, T M ADAMS D J & HILLE B 1979 Ionic selectivity of end-plate channels. *Biophys J* 25 67a.

EISENMAN G, SANDBLOM J & NEHER, E. 1977 Ionic selectivity saturation, brooding and block in the gramicidin A channel: A preliminary report. In: *Metal-ligand interactions in organic chemistry and biochemistry* (ed B. Pullman and N. Goldfarb), pp. 1-36. D. Reidel Publishing Co. Dordrecht, Holland.

EISENMAN G, SANDBLOM J & NEHER, E. 1978. Interactions in cation permeation through the gramicidin channel. Cs, Rb, K, Na, Li, Tl, H and effects of anion binding. *Biophys J* 22: 307-340.

FELLER, W. 1967 *An introduction to probability theory and its applications*, vol 1 3rd ed John Wiley New York.

FERRY J D 1936. Statistical evaluation of sieve constants in ultrafiltration. *J Gen Physiol* 20: 95-104

FINKELSTEIN A. 1974 Aqueous pores created in thin lipid membranes by the antibiotics nystatin, amphotericin B and gramicidin A. Implications for pores in plasma membranes. In: *Drugs and transport processes* (ed B. A. Callingham) pp. 241-250 University Park Press Baltimore Md

FINKELSTEIN A. & HOLZ, R. 1973 Aqueous pores created in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. In: *Membranes. Vol. 2. Lipid bilayers and antibiotics* (ed G. Eisenman) pp. 377-408. M. Dekker New York.

FINKELSTEIN A. & MAURO A. 1963 Equivalent circuits as related to ionic systems. *Biophys J* 3 15-237

FINKELSTEIN A. & ROSENBERG P A. 1979 Single-file transport: Implications for ion and water movement through gramicidin A channels. In: *Membrane transport processes*, vol 3 (ed. C. F. Stevens and R. W. Telen) pp. 73-88 Raven Press, New York.

GOLDMAN D E. 1944 Potential, impedance, and rectification in membranes. *J Gen Physiol* 27 37-60.

HAGIWARA, H & TAKAHASHI K. 1974b The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J Mem Biol* 18. 61-80

HÄGGLUND J ENOS H & EISENMAN G 1979 Multi-site multi-barrier multi-occupancy models for the electrical behavior of single filing channels like those of gramicidin. *Brain Res Bull* 4 154-158.

HECKMAN K. 1955 Zur Theorie der single file Diffusion. I. *Z Phys Chem N F* 44 184-203

HECKMAN K. 1965b Zur Theorie der single file Diffusion. II. *Z Phys Chem N F* 46. 1-35

HECKMANN K & VOLLMERHAUS, W 1970 Zur Theorie der single file Diffusion. IV Vergleich von Leerstellendiffusion und knock-on Mechanismus. *II Phys Chem* 71 320-328

HILLE, B 1975 Ion selectivity saturation, and block in sodium channels. *J Gen Physiol* 66. 535-560

HILLE, B 1975a Ionic selectivity of Na and K channels in nerve membranes. In: *Membranes. Vol 3 Lipid bilayers and biological membranes: dynamic properties* (ed G. Eisenman) pp. 255-323 M. Dekker New York.

HILLE, B 1978. Ionic channels in excitable membranes Current problems and biophysical approaches. *Biophys J* 22. 283-294

HILLE, B & SCHWARZ, W 1978 Potassium channels as multi-ion single-file pores. *J Gen Physiol* 72. 409-442

HLADKY S B & HAYDON D A 1972. Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochim Biophys Acta* 274 294-312.

HODOKIN A. L. & HUXLEY A F 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol* 117 500-544

HODGKIN A. L. & KATZ, H 1949 The effect of sodium ions on the electrical activity of the giant axon of the squid. *J Physiol* 108. 37-77

HODOKIN A. L. & KEYNES R. D 1955 The potassium permeability of a giant nerve fibre. *J Physiol* 128 61-88.

KOEPPE, R. E. II HODGSON K O & STRYER L. 1978. Helical channels in crystals of gramicidin A and of a cesium-gramicidin A complex: an X-ray diffraction study. *J Mol Biol* 121 41-54

LÄUGER, P. 1973 Ion transport through pores: a rate theory analysis. *Biochim Biophys Acta* 311 423-441

LÄUGER, P. 1976. Diffusion-limited ion flow through pores. *Biochim Biophys Acta* 455 493-509

LEVITT H H 1978a Electrostatic calculations for an ion channel I. Energy and potential profiles and interactions between ions. *Biophys J* 22 209-219

LEVITT D H 1978b Electrostatic calculations for an ion channel II Kinetic behavior of the gramicidin A channel. *Biophys J* 22: 221-48

- LEVITT D G, ELIAS S. R. & HAUTMAN J M 1978. Number of water molecules coupled to the transport of sodium, potassium and hydrogen ions via gramicidin nonactin or valinomycin. *Biochim Biophys Acta* 512: 436-451.
- MARKIN V W & CHIZMADIEV Y A. 1974. Induced ionic transport. 1-251. Nauka, Moscow. (In Russian.)
- McBRIDE, II & SZABO G. 1978. Blocking of gramicidin channel conductance by Ag<sup>+</sup>. *Biophys J* 21: 23a.
- MYERS V B & HAYDON D A. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity. *Biochim Biophys Acta* 274: 313-322.
- NEHER, E. 1975. Ionic specificity of the gramicidin channel and the Thallous ion. *Biochim Biophys Acta* 401: 540-544.
- NEHER, E., SANDBLOM, J. & EISENMAN G. 1978. Ionic selectivity, saturation and block in gramicidin A channels. II. Saturation behavior of single channel conductances and evidence for the existence of multiple binding sites in the channel. *J Mem Biol* 40: 97-116.
- PARLIN R. B. & EYRING H. 1954. Membrane permeability and electrical potential. In: *Ion transport across membranes* (ed. H. T. Clarke) pp. 103-118. Academic Press, New York.
- PROCOPIO J. & ANDERSEN O S. 1979. Ion tracer fluxes through gramicidin A modified lipid bilayers. *Biophys J* 25: 8a.
- ROSENBERG P. A. & FINKELSTEIN A. 1978a. Interactions of ions and water in gramicidin A channels. Streaming potentials across lipid bilayer membranes. *J Gen Physiol* 72: 327-340.
- ROSENBERG P. A. & FINKELSTEIN A. 1978b. Water permeability of gramicidin A-treated lipid bilayer membranes. *J Gen Physiol* 73: 341-390.
- SANDBLOM J., EISENMAN G. & NEHER, E. 1977. Ionic selectivity, saturation and block in gramicidin A channels: I. Theory for the electrical properties of ion selective channels having two pairs of binding sites and multiple conductance states. *J Mem Biol* 31: 383-417.
- SARGES R. & WITKOPF B. 1965. Gramicidin V. The structure of valine- and isoleucine-gramicidin A. *J Am Chem Soc* 87: 2011-2020.
- SCHAGINA L. V., GRINFELDT A. E. & LEV A. A. 1978. Interaction of cation fluxes in gramicidin A channels in lipid bilayer membranes. *Nature* 273: 243-245.
- TREGOLD R. H. & JONES R. 1979. A study of gramicidin using deuterium oxide. *Biochim Biophys Acta* 550: 543-545.
- URBAN B. W., HLADKY S. B. & HAYDON D. A. 1978. The kinetics of ion movements in the gramicidin channel. *Fed Proc* 37: 2629-2632.
- URBAN B. W. & HLADKY S. B. 1979. Ion transport in the simplest single file pore. *Biochim Biophys Acta* 554: 410-429.
- URRY D. W., LONG M. M., JACOBS M. & HARRIS, R. D. 1975. Conformation and molecular mechanisms of carriers and channels. *Annals N Y Acad Sci* 264: 203-220.
- USSING H. H. 1949. The distinction by means of tracers between active transport and diffusion. The transfer of iodide across the isolated frog skin. *Acta Physiol Scand* 19: 43-56.
- VARGAS, F. F. 1968. Water flux and electrokinetic phenomena in the squid axon. *J Gen Physiol* 51: 1225-1305.
- VRATCH W. & STRYER, L. 1977. The dimeric nature of the gramicidin A trans-membrane channel. Conductance and fluorescence energy transfer studies of hybrid channels. *J Mol Biol* 113: 89-102.



## Modulation of alamethicin induced conductance by membrane composition

RAMON LATORRE and JAMES J. DONOVAN

Department of Physiology Harvard Medical School, Boston, Mass. 02115 USA

LATORRE, R. & DONOVAN J. J. Modulation of alamethicin-induced conductance by membrane composition. *Acta Physiol Scand* 1980 Suppl 481 37-45. Department of Physiology Harvard Medical School Boston, Mass. 02115 USA.

The effect of cholesterol on the opening and closing of the alamethicin-channel was studied varying the cholesterol content of glycerolmonooleate membranes. Increasing the cholesterol mole fraction in the membrane shifted the log of the steady-state conductance-voltage curve to the right along the voltage axis. The shift was found to be 80-100 mV when the cholesterol mole fraction was increased from 0 to 0.5. The kinetics for the approach to steady-state conductance during voltage clamp was also studied in membranes under these conditions. At all the different cholesterol contents the on kinetic is well described by a single exponential. The off kinetic, on the other hand, is described by double exponential time course. At the same voltage the time constant describing the on current relaxation is the same as the one found for the slow relaxation present in the off relaxation. Increasing the membrane cholesterol content increases the magnitude of the time constant describing the fast and slow process by several orders of magnitude. Cholesterol also increases the voltage dependence of the slow time constant. The effect of cholesterol on the steady-state conductance can be explained, but not in a unique way, by an increase in the membrane dipole potential. Phenomenological comparison of the fast and slow kinetic processes seen in multi-channel membranes with single channel characteristics indicates that: (i) cholesterol increases the mean life of the single channel and (ii) cholesterol increases the mean life of the different conductance levels.

As pointed out by Hodgkin & Huxley (1952) voltage-dependent conductances (permeabilities) should involve the movement of charged or dipolar molecules in the membrane field. "Gating currents" due to this charge movement have been actually observed in several biological preparations, and are thought to be due to the molecular events preceding the opening of the sodium channel (Armstrong & Bezanilla 1974; Keynes & Rojas 1974; Nonner et al 1978; Bullock & Schauf 1978). Several fanciful models of the sodium channel have been proposed but whatever the actual structure of the channel is the movement of the voltage sensor (gating particles) should be affected by changes in membrane composition. For example, alterations in lipid composition may affect the extent of membrane depolarization necessary to open the channels and/or the rates at which the channels open and close. At present, it is very difficult to study the effect of lipid composition on voltage-dependent conductance in

biological membranes because modification of the lipid composition of those membranes is not an easy task and also because the molecular architecture of a "natural" voltage-dependent channel is not known.

In order to provide an insight into the mechanisms by which membrane composition modulates the opening and closing of voltage-dependent channels, we have studied alamethicin-treated planar lipid bilayer membranes.

The ideal system in which to study the effects of membrane composition on channel gating is one in which the membrane composition is well defined and in which the mode of action and molecular structure of the ionophore are well understood. Alamethicin-treated glycerolmonooleate-cholesterol membranes approach this ideal. Alamethicin is a well characterized polypeptide both from the point of view of its molecular structure and from its mode of action in bilayers. On the other hand,



glycerolmonooleate membranes have been used extensively in bilayer studies and the influence of cholesterol on ion transport in this type of membranes has been well characterized (e.g. Gordon & Haydon 1972; Szabo 1974).

Alamethicin is a linear polypeptide antibiotic isolated from *Trichoderma viride* consisting of 19 aminoacids of which 14 are hydrophobic (e.g. Martin & Williams 1976; Pandey et al. 1977). The main fraction of natural alamethicin is the so-called R<sub>30</sub> fraction containing one titratable group with a pK of 5.5 which may correspond to the free glutamine or glutamic carboxyl end (Payne et al. 1970; Pandey et al. 1977).

Mueller & Rudin (1968) were the first to show that alamethicin is able to endow lipid bilayer membranes with a strongly voltage-dependent conductance. The alamethicin-induced conductance (*G*) increases *e*-fold every 4–6 mV similar to the maximum sodium and potassium conductance in nerve and is also strongly dependent on both salt and alamethicin concentrations. Restricting ourselves to positive potentials the conductance can be represented by

$$G = g_0 [Ala]^s [salt] \exp(\alpha e V / kT) \quad (1)$$

where *g*<sub>0</sub> is a constant, *V* is the applied potential and *e*, *k* and *T* have their usual meanings. In phosphatidylethanolamine-decane and in glycerolmonooleate-hexadecane membranes *s* ≈ 9, *s* ≈ 4 and *α* ≈ 6 (Eisenberg et al. 1973; Gordon & Haydon 1975).

Alamethicin increases the conductance of planar lipid bilayer membranes by the formation of pores which do not have a fixed conductance but rather fluctuate between several conductance states (Gordon & Haydon 1977; Eisenberg et al. 1973; Bohem 1974). The opening and closing of these pores are voltage-dependent. The different conductance states are not integral multiples of a unit conductance and have mean life times in the range of milliseconds whereas the mean life time of the pore may span several seconds (Gordon & Haydon 1977; 1975; 1976; Eisenberg et al. 1973; Bohem 1974; Hall 1975; Bohem & Kolb 1978; Kolb & Bohem 1978).

We present here the results of the effect that increasing the mole fraction of cholesterol in glycerolmonooleate membranes has on the alamethicin-induced conductance. Inasmuch as the solvent in solvent-containing membranes may obscure

any effect of lipid composition on this voltage dependent conductance we have minimized the amount of solvent in the membrane by forming the bilayers by apposition of two monolayers (Montal & Mueller 1972). The results are discussed in terms of the different models proposed for the alamethicin channel.

## MATERIALS AND METHODS

The procedure for forming the membrane by apposition of two lipid monolayers, as well as the circuit for measuring the electrical properties of the membranes, has been reported previously (Donovan & Latorre 1979). The lipids used [glycerolmonooleate (GMO, Nu-Chek Prep., Elysian, Minn.) Cholesterol (Chol, Ampec Co., Ann Arbor, Mich.) and phosphatidylethanolamine (PE, Serpico, Bellefonte, Penn.)] were stored at -50°C in CHCl<sub>3</sub> solutions. A portion of this stock solution was evaporated under a stream of nitrogen and redissolved in pentane at a concentration of 1–5 mg/ml. These lipid solutions were prepared fresh every day. GMO-Chol mixtures were prepared by mixing the appropriate volumes of the chloroform-lipid solution with the subsequent evaporation of the CHCl<sub>3</sub> and redissolution in pentane to a final total lipid concentration of 1–5 mg/ml.

### Steady-state experiment

Our experiments were performed with the pure R<sub>30</sub> fraction of alamethicin which was kindly supplied to us by Dr J. E. Hall. After membrane formation the antibiotic was added to only one aqueous compartment (*cis* side). The *cis* side is defined as the positive side of the membrane, and positive current is the flow of cations from the *cis* to the *trans* side. As discussed in detail by Bohem & Kolb (1978), the behavior of the alamethicin system depends, to a certain extent, on the pretreatment with respect to membrane voltage and temperature. We also found that the steady-state conductance drifted for about one hour before stabilizing; therefore, in all experiments, current measurements were not begun until at least one hour after the addition of alamethicin. To obtain the dependence of conductance on alamethicin concentration membranes were formed in 1 M NaCl and a small amount of alamethicin was added. After one hour the conductance was measured at different potentials, and another small addition of alamethicin was made. This procedure was repeated after each new addition of alamethicin. To obtain the dependence of the conductance on salt concentration the membrane was formed in 20 mM NaCl, alamethicin was added and, after one hour the conductance measured. Small portions of a 6 M NaCl solution (unbuffered pH ~5.5) were added symmetrically to the membrane and the conductance measured each time.

### Kinetics

To measure the kinetic response of multi-channel membranes to a voltage pulse, membranes were formed in 1 M

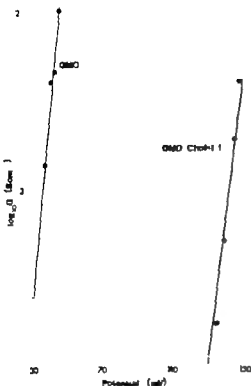


Fig. 1 Steady-state conductance-voltage curves for the alamethicin-induced conductance in GMO and GMO-Chol (molar ratio 1:1) membranes: the conductance increases fold every 6 mV in both cases  $[Ala]=600$  ng/ml,  $[NaCl]=1.0$  M,  $T=22\pm1^\circ\text{C}$ .

NaCl and 8.30  $\mu\text{M}$  alamethicin was added. After one hour voltage pulses were applied starting from low potentials and proceeding to high potentials. The order was then reversed. No difference was seen between two such pulses of the same potential. Generally from 4 to 3, current relaxations were scanned to improve the signal to noise ratio.

## RESULTS AND DISCUSSION

### General

The voltage-dependent ion conductance induced by alamethicin has been the subject of several studies (e.g. Bohelm 1978; Hall 1978) but a systematic study of the effect of lipid composition has not been done. Gordon & Haydon (1975) have reported an effect of lipid composition in solvent-containing bilayers on the rate constant for the transitions between the different conductance level of the alamethicin channel. They found that the rate constants are very sensitive to the lipid composition, but the only conclusion that they were able to draw from

their results was that membrane "fluidity" is involved in some way.

At least two problems must be considered when comparing effects of lipid composition on carrier and channel mediated transport in solvent-containing membranes. First, Pagano et al (1977) have shown that the bilayer composition of GMO-cholesterol-decane membranes is different than the composition of the membrane-forming solution such that the molar ratio of cholesterol to GMO in the membrane is about one half of that in the membrane-forming solution. Second, the ratio of lipid/hydrocarbon solvent in the bilayer appears to be a function of the lipid concentration and the membrane-forming solution (Waldbüßig & Szabo 1978). We think, therefore, that a comparison between the mode of action of an ionophore in solvent-containing bilayers of different lipid composition should be viewed with caution as they may contain widely different amounts of solvent.

Because they contain very small amounts of solvent, membrane made by apposition of two monolayers are more suitable for this kind of study (e.g. Reyes & Latorre 1979). Further indirect evidence will be given below that indicates that the bilayers formed with this method actually have approximately the same composition as the monolayers from which they are formed.

### Changes in steady-state alamethicin-induced conductance due to the presence of cholesterol in the bilayer

Fig. 1 shows the effect of increasing cholesterol content of GMO membranes on the alamethicin-induced conductance. As can be seen in the figure cholesterol shifts the  $\log G$  vs.  $V$  curve to the right along the voltage axis by more than 100 mV. Thus, at constant salt and alamethicin concentration in the aqueous phases, the voltage needed to open a given number of channels is increased with increasing cholesterol. It is apparent from Fig. 1 that cholesterol does not change the voltage dependence of the conductance induced by alamethicin. The effect of increasing the mole fraction of cholesterol in GMO-Chol membranes is summarized in Table 1. The proportionality constant  $g$  in equation 1 sets the magnitude of  $G$  at given values of  $[Ala]$ ,  $[salt]$  and  $V$ . For convenience we set these at  $[Ala]=1000$  ng/ml,  $[salt]=1$  M and  $V=100$  mV. The results shown in Table 1 indicate that changing the chole-

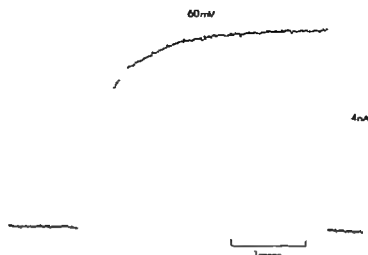


Fig. 2 The alamethicin-induced current in a GMO membrane in response to a voltage step from zero to +60 mV. The slight delay is not visible in this picture.  $[Ala]=250$   $\mu$ M,  $[NaCl]=1$  M. Membrane area:  $4 \times 10^{-4}$   $cm^2$ .  $T=22 \pm 1^\circ C$ .

sterol content of GMO membranes has little or no effect on the voltage dependence of the conductance and it only affects the absolute magnitude of the conductance.

The large change in  $g_s$  ( $\sim 10$ -fold) may have a number of conceivable causes. First variations in membrane lipid composition may change the adsorption coefficient of alamethicin in the membrane i.e. due to the high power dependence of the conductance on alamethicin concentration, a 10-fold change in adsorption coefficient would be enough to explain the change in  $g_s$ . Second

changes in membrane viscosity could contribute to the change in  $g_s$ . It is known (Finkelstein & Cass 1967) that cholesterol lowers the water permeability of lecithin bilayers by about 10-fold. Under our experimental conditions Donovan (1979) found that the acetamide permeability decreases by a factor of 6 when the mole fraction of cholesterol in the membrane changes from 0 to 0.5 (GMO membranes). Third the channel formation may be impaired for electrostatic reasons. Szabo (1974) has shown that increasing the mole fraction of cholesterol in GMO bilayers increases the lipophilic anion-induced conductance but decreases the lipophilic cation-induced conductance. Inasmuch as GMO and cholesterol are neutral molecules at the pH used in Szabo's experiments the most likely explanation for his results is that cholesterol increases the dipole potential of the membrane by as much as 100 mV. We have performed similar experiments on membranes made by apposition of two monolayers. Our results indicate that cholesterol increases the dipole potential in these membranes as well. However in contrast to Szabo's results we find that the conductance changes for anions and cations are not symmetric: the increase in anion conductance is less than the decrease in cation conductance at any given fraction of cholesterol in the membrane. This indicates that the dipole potential is not the only factor determining the conductance changes. Other factors, such as mem-

Table 1. Parameters of steady state alamethicin induced conductance in GMO and GMO-Chol membranes.

The parameters describe the steady-state alamethicin-induced conductance according to the equation:

$$G = g_s C^{\delta} \exp(aeV/kT)$$

The membrane area was  $1 \times 10^{-4}$   $cm^2$ .

Lipid	$g_s$ (nS)	$\delta$	$a$	$\alpha$
GMO	$3.9 \times 10^6$	$11.6 \pm 1$	$5.9 \pm 0.5$	$4.4 \pm 0.5$
80% GMO- 20% Chol	$7.9 \times 10^6$	$12.3 \pm 1$		$4.1 \pm 0.5$
70% GMO- 30% Chol	$2.8 \times 10^6$			$4.4 \pm 0.6$
50% GMO- 50% Chol	$1.8 \times 10^6$	$10.6 \pm 1$	$5.2 \pm 0.7$	$4.1 \pm 1.0$

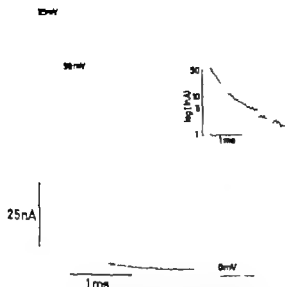


Fig. 3 The "off" response of the alamethicin-induced current in a GMO membrane. A voltage pulse of 115 mV was applied, and the current allowed to come to steady state. The figure shows the end of the 115 mV pulse. The potential was then stepped to 0 mV. Finally the potential was stepped to 0 mV. The inset shows a semi-log plot of current versus time. Two exponentials are apparent, with  $\tau = 0.3$  ms and  $\tau = 0.85$  ms. The amplitude of the fast relaxation is 36 nA and that of the slow relaxation is 24 nA. Membrane area:  $2 \times 10^{-4}$  cm<sup>2</sup>.

brane viscosity as suggested by the acetamide permeability experiments must also be involved.

Steady-state measurements alone cannot distinguish between the different possibilities given above and therefore we have performed kinetic measurements in order to provide a further insight into the mechanism(s) by which cholesterol alters the alamethicin-induced conductance.

#### Effect of cholesterol on the kinetic characteristics of the alamethicin channel

The record in Fig. 2 taken in an alamethicin-treated GMO membrane shows that if the voltage is suddenly changed from 0 to a sufficient positive value, the alamethicin-induced current increases monotonically to a steady state value. The time-course of the current is well described by a single exponential with a time constant ( $\tau$ ) of 0.4 ms. Exponential time courses for the alamethicin induced current have been reported previously for alamethicin-treated PE and phosphatidylcholine membranes

can also be obtained from the alamethicin "off" kinetics. Fig. 3 shows the alamethicin "off" kinetics in a GMO membrane. The relaxation consists of an "instantaneous" ohmic drop in current and the time course of the current drop that follows the ohmic drop is well described by two exponentials, a fast relaxation with time constant  $\tau$  and a slow relaxation with time constant  $\tau_s$ . In GMO membranes  $\tau$  is about 6–10-fold greater than  $\tau_s$ .

Increasing the cholesterol content of the membranes has a great effect on both  $\tau$  and  $\tau_s$ . In Fig. 4

is plotted as a function of potential for membranes made of GMO-80%GMO-20%Chol and 50%GMO-50%Chol  $\tau$  was obtained from both on and off relaxations. Fig. 4 shows that increasing cholesterol has two effects on the time constant for the slow relaxation. First, it increases the magnitude of  $\tau$  and second, it increases the voltage-dependence of  $\tau$ . Cholesterol also slows down the fast time constant (Fig. 5) but it is difficult with the present data to extract a definitive conclusion about whether cholesterol is also modifying the voltage-dependence of the fast process. It is noteworthy that both  $\tau$  and  $\tau_s$  are independent of alamethicin concentration (Fig. 4 and 5).

Recently both Bohm & Kolb (1978) have presented a detailed analysis of the alamethicin kinetics in dioleoylphosphatidylcholine-decane membranes. They made a comparison between the ob-

The calculated dipole potential change in bilayers made from monolayers is 80 mV when the cholesterol mole fraction in the lipid solution used to form the monolayers is increased from 0 to 0.5. This calculation has been done assuming that ions of similar structure like TPhAs and TPhB<sup>+</sup> will be affected to the same extent by changes in membrane fluidity. For the same change in cholesterol mole fraction in the membrane forming solution, Szabo (1974) obtained a dipole potential change of 30 mV in GMO-decane membranes. This result is to be expected if the membrane cholesterol mole fractions in Szabo membranes were about half of that of our membranes. In view of the Pagano et al. (1972) results and in view of the differences in dipole potential changes obtained by Szabo and us, the cholesterol content in our membranes appears to be closely related to that of the membrane forming solution.

Batterson & Mueller (1974) have reported an S-shape time course for the alamethicin-induced current relaxation. An S-shape time course has also been found by Bohm (1978), but it disappeared after high conductance was induced whereupon the time course became exponential. Our results in GMO and GMO-cholesterol membranes indicate that delay is always present in the current relaxations and takes place in the  $\mu$ s range.

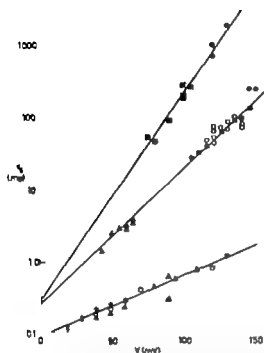


Fig. 4 Plot of  $G$  vs.  $V$  for different mole fractions of cholesterol in GMD membranes and different alamethicin concentrations. The lowest curve is for pure GMD membranes the middle curve is for 80% GMD-20% Cholesterol and the upper curve is for 50% GMD-50% Cholesterol. Both on and off relaxations are represented. The different alamethicin concentrations are:  $\square=33$  ng/ml  $\circ=100$  ng/ml  $\bullet=200$  ng/ml  $\blacktriangle=770$  ng/ml  $\blacktriangle=300$  ng/ml  $\blacklozenge=400$  ng/ml  $\bullet=600$  ng/ml  $\bullet=900$  ng/ml and  $\blacksquare=1600$  ng/ml. Note that  $G$  depends on potential and cholesterol concentration, but not on alamethicin concentration.  $[NaCl]=1$  M.

served dependence of  $\tau_1$  and  $\tau$  on voltage and temperature with the results of the analysis of long-lasting single channels. Such a phenomenological comparison indicates that  $\tau$  correlates well with the lifetime of single channels, and is taken to be due to the change in the number of channels present. The fast relaxation process, on the other hand, appears to arise from a shift in the probability distribution of the different conductance levels as the potential is changed.  $\tau_1$  correlates well with the lifetime of the different conductance levels.

We have made such a correlation for GMD-Chol membranes containing a cholesterol mole fraction of 0.5. Fig. 11 shows a record of conductance fluctuations arising from a single alamethicin channel at an applied voltage of 190 mV. It is apparent that the mean life-time of a single pore is on the same order as  $\tau$  (see Fig. 4) and that the mean half-life of the

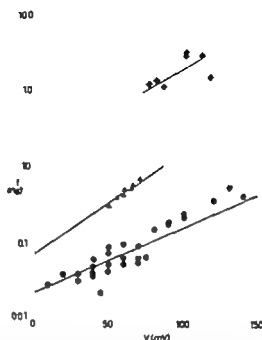


Fig. 5 Plot of  $G$  vs.  $V$  for different mole fractions of cholesterol in GMD membranes. The lowest curve is for pure GMD the middle curve for 80% GMD-20% Cholesterol, and the upper curve for 50% GMD-50% Cholesterol.  $[NaCl]=1$  M. Two or more concentrations of alamethicin are represented for each cholesterol mole fraction.

different conductance levels (bottom of Fig. 6) is on the same order as  $\tau_1$  (see Fig. 5). Therefore in terms of the single alamethicin channel, cholesterol increases the channel lifetime, increases the dependence of channel lifetime on potential and increases the mean half-life of the different conductance levels.

As stated above, the change in the steady-state alamethicin-induced conductance promoted by cholesterol could be accounted for by a decrease in the alamethicin adsorption coefficient, an increase in membrane viscosity, an increase in the membrane dipole potential, or by changes in all these parameters simultaneously. Taken together the steady-state data and the kinetic data allow us to

Changes in dipole potential can affect the alamethicin gating since we can view the alamethicin molecules as dipoles moving in an electric field, the positive end of which is pushed into the membrane when the field is applied. Addition of cholesterol will increase the height of the energy barrier across which the positive end of the alamethicin molecules must move. Yarranton et al. (1977) have calculated the dipole moment of alamethicin to be of the order 67 Debyes.

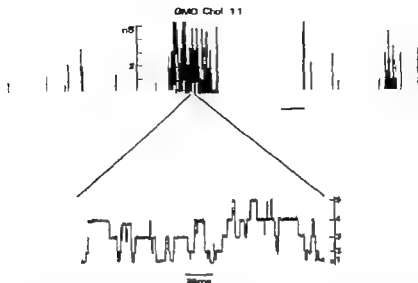


Fig. 6 Current bursts due to single channels of alamethicin. The expanded time scale below shows the different conductance levels more clearly, and the scale on the right marks off the different levels. In this particular record up to six conductance levels can be seen. 50%GMO-50%Cholesterol membrane.  $[Ala]=250$  ng/ml,  $[NaCl]=1$  M. Applied potential, 190 mV. Temperature:  $22 \pm 1^\circ\text{C}$ .

discuss in some detail each of these possibilities. Thus, the 3-fold change in the  $\tau$  at zero potential (see Table 2) is consistent with a change in the membrane viscosity, but it would not explain why cholesterol increases the voltage dependence of  $\alpha$ . The shift in the  $\log G$  vs.  $V$  curve (Fig. 1) is also difficult to explain in terms of fluidity since we would expect that changes in membrane viscosity would slow down both the rates of channel formation ( $\mu$ ) and channel closing ( $\lambda$ ). Since the steady state conductance is given by Eisenberg et al. (1973)

$$G = \frac{\mu}{\lambda}$$

one would expect no change in the total number of open channels. In other words, a change in membrane viscosity will change the activation energy barriers for channel opening and closing, but will not change the relative energies of the open and closed states.

Either an increase in dipole potential or a change in the alamethicin adsorption coefficient may explain the change in  $g$  (Table 1). At present, we cannot discard either of these possibilities since we do not have independent measurements of the alamethicin partition coefficients as a function of cholesterol concentration. However, if we assume that the change in  $g$  is primarily due to a dipole poten-

tial change then we are forced to locate the open configuration somewhere in the middle of the membrane. Taking into account this consideration together with the voltage dependence of the alamethicin-induced conductance, we have calculated that the change in dipole potential felt by alamethicin in a 50%GMO-50%Chol membrane relative to a GMO membrane is about 80 mV. This value is in good agreement with the value obtained using lipophilic ions as probes of changes in dipole potential (Donovan 1979).

#### Membrane composition and possible modes of pore formation

The high power dependence of the conductance on the alamethicin concentration, and the multi-state

Table 2. Alamethicin parameters for membranes of different composition

Membrane	$\tau$ (ms)	$\alpha^*$	$\alpha^*_h$	$\alpha'$
GMO	0.09	4.4	0.51	3.9
80%GMO-20%Chol	0.25	4.1	1.1	3.0
50%GMO-50%Chol	0.27	4.1	1.68	2.5

Calculated from steady-state conductance data. Defined according to the equation  $\alpha = \exp(\alpha^* eV/kT)$ . Calculated according to  $\alpha_h = \alpha - \alpha'$ .

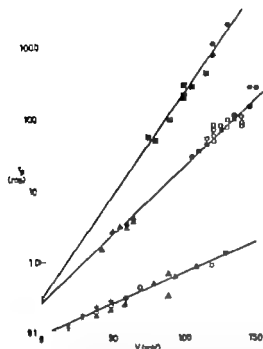


Fig. 4 Plot of  $\tau$  vs.  $V$  for different mole fractions of cholesterol in GMO membranes and different alamethicin concentrations. The lowest curve is for pure GMO membranes, the middle curve is for 80% GMO-20% Cholesterol and the upper curve is for 50% GMO-50% Cholesterol. Both on and off relaxations are represented. The different bicin concentrations are:  $\circ=33$  ng/ml  $\square=100$  ng/ml  $\triangle=200$  ng/ml  $\diamond=770$  ng/ml  $\nabla=300$  ng/ml  $\star=400$  ng/ml  $\ast=600$  ng/ml  $\otimes=900$  ng/ml and  $\blacksquare=1600$  ng/ml. Note that  $\tau$  depends on potential and cholesterol concentration, but not on alamethicin concentration.  $[NaCl]=1$  M.

served dependence of  $\tau_1$  and  $\tau$  on voltage and temperature with the results of the analysis of long-lasting single channels. Such a phenomenological comparison indicates that  $\tau$  correlates well with the lifetime of single channels and is taken to be due to the change in the number of channels present. The fast relaxation process on the other hand appears to arise from a shift in the probability distribution of the different conductance levels as the potential is changed.  $\tau_1$  correlates well with the lifetime of the different conductance levels.

We have made such a correlation for GMO-Chol membranes containing a cholesterol mole fraction of 0.5. Fig. 6 shows a record of conductance fluctuations arising from a single alamethicin channel at an applied voltage of 190 mV. It is apparent that the mean life-time of a single pore is on the same order as  $\tau$  (see Fig. 4) and that the mean half-life of the

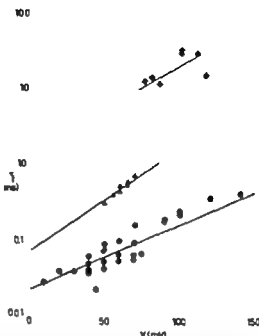


Fig. 5 Plot of  $\tau$  for different mole fractions of cholesterol in GMO membranes. The lowest curve is for pure GMO the middle curve for 80% GMO-20% Cholesterol and the upper curve for 50% GMO-50% Cholesterol.  $[NaCl]=1$  M. Two or more concentrations of alamethicin are represented for each cholesterol mole fraction.

different conductance levels (bottom of Fig. 6) is on the same order as  $\tau_1$  (see Fig. 5). Therefore in terms of the single alamethicin channel cholesterol increases the channel lifetime increases the dependence of channel lifetime on potential and increases the mean half life of the different conductance levels.

As stated above the change in the steady state alamethicin-induced conductance promoted by cholesterol could be accounted for by a decrease in the alamethicin adsorption coefficient, an increase in membrane viscosity, an increase in the membrane dipole potential<sup>8</sup> or by changes in all these parameters simultaneously. Taken together the steady state data and the kinetic data allow us to

Changes in dipole potential can affect the alamethicin gating since we can view the alamethicin molecules as dipoles moving in an electric field: the positive end of which is pushed into the membrane when the field is applied. Addition of cholesterol will increase the height of the energy barrier across which the positive end of the alamethicin molecules must move. Yamamoto *et al.* (1977) have calculated the dipole moment of alamethicin to be of the order 67 Debyes.

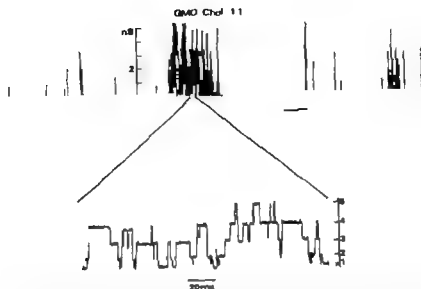


Fig. 6 Current bursts due to single channels of alamethicin. The expanded time scale below shows the different conductance levels more clearly and the scale on the right counts off the different levels. In this particular record up to its conductance levels can be seen. 40%GMO-60%Cholesterol membrane. [Ala]=250 ng/ml, [NaCl]=1 M. Applied potential 190 mV. Temperature:  $22 \pm 1^\circ\text{C}$ .

discuss in some detail each of these possibilities. Thus, the 3-fold change in the  $\tau$  at zero potential ( $\tau_0$ , see Table 2) is consistent with a change in the membrane viscosity but it would not explain why cholesterol increases the voltage dependence of  $\tau$ . The shift in the log  $G$  vs.  $V$  curve (Fig. 1) is also difficult to explain in terms of fluidity since we would expect that changes in membrane viscosity would slow down both the rates of channel formation ( $\mu$ ) and channel closing ( $\lambda$ ). Since the steady state conductance is given by Eisenberg et al. (1973)

$$G = \frac{\mu}{\lambda}$$

one would expect no change in the total number of open channels. In other words a change in membrane viscosity will change the activation energy barriers for channel opening and closing, but will not change the relative energies of the open and closed states.

Either an increase in dipole potential or a change in the alamethicin adsorption coefficient may explain the changes in  $g_s$  (Table 1). At present, we cannot discard either of these possibilities since we do not have independent measurements of the alamethicin partition coefficients as a function of cholesterol concentration. However if we assume that the change in  $g_s$  is primarily due to a dipole poten-

tial change, then we are forced to locate the open configuration somewhere in the middle of the membrane. Taking into account this consideration together with the voltage dependence of the alamethicin-induced conductance we have calculated that the change in dipole potential felt by alamethicin in a 50%GMO-50%Chol membrane relative to a GMO membrane is about 80 mV. This value is in good agreement with the value obtained using lipophilic ions as probes of changes in dipole potential (Donovan 1979).

#### Membrane composition and possible modes of pore formation

The high power dependence of the conductance on the alamethicin concentration, and the multi-state

Table 1. Alamethicin parameters for membranes of different composition

Membrane	(ms)	$n^0$	$n^1$	$n_2$
GMO	0.09	4.4	0.51	3.9
80%GMO-20%Chol	0.25	4.1	1.1	3.0
50%GMO-50%Chol	0.27	4.1	1.68	2.5

Calculated from steady-state conductance data. Defined according to the equation  $\tau = \tau_0 \exp(\alpha V)$  or  $\tau = \tau_0 \exp(\alpha V - \beta V)$ .  
Calculated according to  $n_2 = n^0 - n^1$ .



character of the single alamethicin channels (Fig. 6) suggest that each channel is formed by association of several monomers of alamethicin. However there is not a unique model describing the underlying molecular processes involved in the conductance transitions. Hall (1975) and Gordon & Haydon (1976) have proposed that the different conductance levels arise as a consequence of "conformational" changes of an oligomer containing a fixed number of monomers. Baumann & Mueller (1974), Boheim (1974) and Boheim & Kolb (1978) on the other hand, have proposed the existence of a pore that varies in diameter by uptake and release of monomers.

Boheim & Kolb (1978) have argued strongly against the models of Hall (1975), Gordon and Haydon (1976). Their main line of argument is based on the alamethicin concentration dependence of  $\tau$  in phosphatidylcholine alamethicin-treated membranes. They found that  $\tau$  is well described by the equation

$$\tau = k[\text{Ala}]^2 \exp(2.6 \text{ eV}/kT) \quad (2)$$

where  $k$  is a proportionality constant. This concentration dependence suggests that the pore does not contain a fixed number of monomers.

Our results in GMO and GMO-Chol membranes indicate that  $\tau$  is independent of alamethicin concentration and that the voltage dependence of  $\tau$ , regardless of the cholesterol mole fraction, is always less than that found in PC membranes by Boheim & Kolb (1978). The difference could be ascribed to differences in the lipid composition (GMO vs. PC) or to the presence of solvent in the PC membranes. However preliminary experiments performed by us using PE membranes formed by apposition of two monolayers indicate that  $\tau$  is strongly dependent on alamethicin concentration in this type of bilayer as well. Apparently then, the molecular mechanisms which give rise to the conductance transitions are different in phospholipids than in GMO membranes.

## CONCLUSION

We have shown in this paper that the alamethicin-induced voltage-dependent permeability can be modified to a great extent by the nature of the lipid. In the following, we state the main results and conclusions which are presented in this paper. (a) The voltage necessary to open a given number of alame-

thin channels is increased by increasing the mole fraction of cholesterol in GMO membranes. If the alamethicin channel opens by the process of orientation of the alamethicin molecular dipole in the electric field, the above result can be understood in terms of an increase of the membrane dipole potential induced by cholesterol. The shift to the right of the log G-V curve induced by cholesterol can also be explained in terms of changes in the amount of alamethicin adsorbed, but not by changes in membrane viscosity. (b) In many channel membranes, both the on and the off alamethicin-induced kinetic are altered by cholesterol. Cholesterol slows down the alamethicin-induced kinetic and modified its voltage-dependence. In terms of the single alamethicin channel, the results can be interpreted as an increase in the half life of the channel and a change of the equilibrium position of the closed configuration of the alamethicin channel respectively. (c) Comparison of the kinetic processes describing channel decay in GMO and phospholipid bilayer membranes suggests that the mechanism of formation of the alamethicin channel is different in these two types of membranes.

This work was supported by a NIH grant No. GM 27715.

## REFERENCES

- ARMSTRONG G. M. & BEZANILLA F. 1974. Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. Gen. Physiol.* 65: 533-552.
- BAUMANN G. & MUELLER, P. 1974. A molecular model of membrane excitability. *J. Supramolec. Struct.* 1: 538-557.
- BOHEIM I. 1974. Statistical analysis of alamethicin channels in black lipid membranes. *J. Membr. Biol.* 19: 777-803.
- BOHEIM G. & KOLB H. A. 1978. Analysis of the multi-pore system of alamethicin in a lipid membrane. I. Voltage-jump current-relaxation experiments. *J. Membr. Biol.* 38: 99-150.
- BULLOCK J. H. & SCHAUF C. L. 1978. Combined voltage-clamp and dialysis of myxococcal axons: behavior of membrane asymmetry currents. *J. Physiol. (Lond.)* 278: 309-324.
- DONOVAN J. J. 1979. The effect of membrane composition on the opening and closing of voltage-dependent channels in lipid bilayer membranes. Ph.D. dissertation, University of Chicago. Chicago, IL.
- DONOVAN J. J. & LATORRE, R. 1979. Inactivation of the alamethicin-induced conductance caused by quaternary ammonium ion and local anesthetics. *J. Gen. Physiol.* 73: 425-451.

- EISENBERG M., HALL, J. E. & MEAD C. A. 1973. The nature of the voltage-dependent conductance induced by alamethicin in black lipid membranes. *J Membr Biol* 14 143-176.
- FINKELSTEIN A. & CASS A. 1967. Effect of cholesterol on the water permeability of thin lipid membranes. *Nature (Lond.)* 216, 717-718.
- GORDON L. G. M. & HAYDON D. A. 1972. The unit conductance channel of alamethicin. *Biochim Biophys Acta* 355 1014-1018.
- GORDON L. G. M. & HAYDON D. A. 1975. Potential-dependent conductances in lipid membranes containing alamethicin. *Phil Trans R Soc Lond B* 270 437-447.
- GORDON L. G. M. & HAYDON D. A. 1976. Kinetics and stability of alamethicin conducting channels in lipid bilayers. *Biochim Biophys Acta* 436 541-556.
- HALL, J. E. 1975. Toward molecular understanding of excitable Biophys J 15 934-939.
- HALL, J. E. 1978. Channels in black lipid films. In: *Membrane transport in biology*. Vol. 1. Concepts and models (ed. G. Giebisch, D. C. Tosteson and H. H. Ussing) pp. 475-531. Springer Verlag, Berlin, Heidelberg, New York.
- HODGKIN A. L. & HUXLEY A. F. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol (Lond.)* 117 500-544.
- KEYNES R. D. & ROJAS E. 1974. Kinetics and steady-state properties of the charged system controlling sodium conductance in the squid giant axon. *J Physiol (Lond.)* 239 847-867.
- KOLB H. A. & BOHEIM G. 1978. Analysis of the single-pore system of alamethicin in lipid membrane. II. Auto-correlation analysis and power spectral density. *J Membr Biol* 38 151-191.
- MARTIN D. R. & WILLIAMS R. J. P. 1976. Chemical nature and sequence of alamethicin. *Biochem J* 153 181-190.
- MONTAL, M. & MUELLER, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc Natl Acad Sci USA* 69 3561-3566.
- MUELLER, P. & RUDIN D. O. 1968. Action potentials induced in bimolecular lipid membranes. *Nature (Lond.)* 217 713.
- NONNER, W., ROJAS E. & STAMPFLI K. 1978. Asymmetrical displacement currents in the membrane of frog myelinated nerve: early time course and effects of membrane potential. *Pflügers Arch* 375 75-85.
- PAGANO R. E., RUYSSCHAERT J. M. & MILLER I. R. 1972. The molecular composition of some lipid bilayer membranes in aqueous solution. *J Membr Biol* 10 11-30.
- PANDEY R. C., COOK, J. C. & RINEHART K. L. 1977. High resolution and field absorption mass spectrometry studies and revised structures of alamethicin I and II. *J Amer Chem Soc* 99 8469-8483.
- PAYNE, J. W., JAKES R. & HARTLEY H. S. 1970. The primary structure of alamethicin. *Biochem J* 117 757-766.
- REYES J. & LATORRE, R. 1979. Effect of benzyl alcohol and chloroform on bilayers made from monolayers. *Biophys J* 28 259-280.
- SZABO G. 1974. Dual mechanism for the action of cholesterol on membrane permeability. *Nature* 252 47-49.
- WALDBILLIG R. & SZABO G. 1978. Solvent-depleted bilayer membranes from concentrated lipid solutions. *Nature (Lond.)* 272 839-840.
- YANTORNO R. E., TAKASHIMA, S. & MUELLER, P. 1977. The dipole moment of voltage-dependent conductance producing antibiotic-alamethicin. *Biophys J* 17 67a.



## Effects of lipid structure on the kinetics of carrier mediated ion transport

R. BENZ, D. CROS, K. JANKO, P. LÄUGER and O. STARK

Department of Biology, University of Konstanz, D-7750 Konstanz, Germany

BENZ, R., CROS, D., JANKO, K., LÄUGER, P. & STARK, O. Effects of lipid structure on the kinetics of carrier-mediated ion transport. *Acta Physiol Scand* 1980 Suppl 481 47-52. Department of Biology, University of Konstanz, Germany

The mechanism of alkali-ion transport mediated by valinomycin (or similar macrocyclic carriers) may be studied using artificial planar bilayer membranes. The rate constants of the single transport steps (association and dissociation of the ion-carrier complex, translocation of free and complexed carrier) can be determined from electrical relaxation experiments. The turnover number of valinomycin which may be calculated from the rate constants is found to be  $10^4$ – $10^5$  s<sup>-1</sup>. Carriers of the valinomycin-type offer the possibility of studying the relationship between membrane structure and transport kinetics. Increasing the chain-length of the lipid strongly reduces the translocation rate constants of the free and the loaded carrier, and also (in the case of leaf-like membranes) the association rate constant. Increasing the number of double bonds in the fatty-acid residues of the lipid leads to an increase of the translocation rate constants. These effects are discussed in terms of microviscosity of the membrane. Addition of cholesterol to monoglyceride membranes seems to affect both the microviscosity and the dipolar potential at the membrane-solution interface.

Certain antibiotics such as valinomycin, monactin, or enniatin B have been shown to act as mobile ion carriers which facilitate passive ion transport through lipid bilayer membranes (Ovchinnikov et al 1974). These compounds form complexes with alkali ions in which the ion is surrounded by a cage of oxygen atoms. In the past years, artificial planar bilayer membranes have been extensively used for studying the mechanism of carrier-mediated ion transport (Muehler & Rudin 1967, Andreoli et al 1967, Lev & Buzhinsky 1967, Eisenman et al 1968, Liberman & Topaly 1968, Stark & Benz 1971). From the dependence of membrane conductance on the concentration of potassium and valinomycin it has been concluded that potassium is transported as a 1:1 complex between ion and carrier (Stark & Benz 1971). In the presence of the carrier, ion transport through the membrane-solution interface may occur in either of two ways (Benz et al 1976). The ion-carrier complex may form already in the aqueous solution and may then cross the interface ("solution-complexation mechanism"). Alternatively, a hydrated ion from the aqueous phase may

react in the interface with a carrier molecule located in the lipid ("interfacial-complexation mechanism"). The complex then moves to the other interface where the ion is released to the aqueous solution. If the first mechanism would be operative, diffusion polarization should occur at high current densities, i.e. the carrier should be depleted in the aqueous phase on one side and accumulated at the other. Such a diffusion polarization is never observed in the presence of valinomycin and potassium and this gives strong evidence that in this system the interfacial-complexation mechanism is operative (Stark & Benz 1971). The same conclusion holds true for other cation carriers such as monactin (Stark & Benz 1971) or enniatin B (Benz 1978). An example for the solution-complexation mechanism has been found, however, in the case of a synthetic prolin-containing analog of valinomycin

Permanent address: Laboratoire de Chimie Physique, Université des Sciences et Techniques de Languedoc, F 34060, France

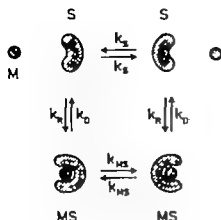


Fig. 1 Transport of ion M mediated by a carrier S.

(Ting-Beall et al. 1974; Benz et al. 1976; Andersen et al. 1977)

#### Electrical relaxation methods for the study of carrier kinetics

According to the interfacial-complexation mechanism the overall transport process occurs in four distinct steps (Fig. 1): (i) association of ion M and carrier S at the interface (rate constant  $k_a$ ); (ii) translocation of the complex MS to the other interface (rate constant  $k_{MS}$ ); (iii) dissociation of the complex MS at the other interface (rate constant  $k_d$ ); and (iv) back-transport of carrier S (rate constant  $k_b$ ). For the study

of the kinetics of this reaction and the determination of the single rate-constants, fast relaxation techniques have to be used. Two different electrical relaxation methods have been applied to carrier systems (Fig. 2). In the voltage-jump current-relaxation method, the voltage across the membrane is quickly changed and the approach of the membrane current towards a stationary value is followed (Stark et al. 1971). A somewhat better time resolution (down to about 200 ns) may be achieved with the so-called charge-pulse technique (Benz & Läuger 1976). In this method the membrane capacitance is charged up to an initial voltage of say 10 mV by a brief current-pulse (Fig. 2). Thereafter the external circuit is switched to virtually infinite resistance and the decay of membrane voltage (caused by ion conduction processes in the membrane) is followed.

#### Determination of the rate constant of the individual transport steps

The analysis of the carrier model represented in Fig. 1 yields the following expression for the time

course of membrane voltage  $V$  in the charge-pulse experiment:

$$V(t) = V_0 [a \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3)] \quad (1)$$

$$a + a_2 + a_3 = 1 \quad (2)$$

where  $V_0$  is the initial voltage. The relaxation times  $\tau_1$ ,  $\tau_2$ ,  $\tau_3$  and the relaxation amplitudes  $a$ ,  $a_2$ ,  $a_3$  are complicated functions of the rate constants (Benz & Läuger 1976). Under favourable experimental conditions where all three relaxation processes can be resolved, the rate constants  $k_a$ ,  $k_b$ ,  $k_{MS}$  and  $k_d$  (Fig. 1) can be determined from records of  $V(t)$ . A similar analysis is possible for voltage-jump current relaxation experiments (Stark et al. 1971).

Rate constants for a number of cation-carrier systems have been evaluated in the last years by these methods. As a specific example we consider the results obtained for valinomycin/Rb<sup>+</sup> in a monolein/n-decane membrane (25°C, 1 M RbCl) (Benz & Läuger 1976):

$$k_a = 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$$

$$k_b = 2 \times 10^5 \text{ s}^{-1}$$

$$k_{MS} = 3 \times 10^5 \text{ s}^{-1}$$

$$k_d = 4 \times 10^4 \text{ s}^{-1}$$

At one-molar concentration of the transported ion ( $c_M = 1 \text{ M}$ ) the rate constants of association ( $k_a$ ), dissociation ( $k_d$ ) and translocation of the loaded carrier ( $k_{MS}$ ) are approximately equal ( $2$ – $3 \times 10^5 \text{ s}^{-1}$ ). The rate-determining step in this system is the back-transport of the free carrier ( $k_b = 4 \times 10^4 \text{ s}^{-1}$ ).  $k_{MS} = 3 \times 10^5 \text{ s}^{-1}$  is the frequency of jumps of an

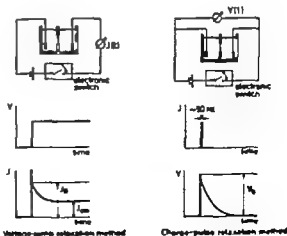


Fig. 2 Electrical relaxation method for the study of ion transport kinetics in planar bilayer membranes.

Table 1 Rate constants of valinomycin-mediated Rb<sup>+</sup> transport through membranes made from phosphatidylcholines (PC) or  $\alpha$ -monoglycerides (G) with different length of the mono-unsaturated fatty acid residue: palmitoleyl (16:1) oleoyl (18:1)  $\Delta^5$ -eicosenoyl (20:1) erucyl (22:1) and nervonoyl (22:1) (Benz et al 1973, Bent et al 1977)

The solvent for membrane formation was *n*-decane;  $T=25^\circ\text{C}$ . Data for monoglycerides have been obtained from charge-pulse experiments, data for phosphatidylcholines from voltage-jump or (denoted by an asterisk) from charge-pulse experiments

Lipid	$k_a/10^4 \text{ M}^{-1} \text{ s}^{-1}$	$k_t/10^4 \text{ s}^{-1}$	$k_{\text{off}}/10^4 \text{ s}^{-1}$	$k_d/10^4$
d(16:1)-PC	4.3(8.2*)	45	9.1	1.3(2.2*)
d(18:1)-PC	2.1	—	—	1.1
d(22:1)-PC	0.26	—	—	0.4
d(24:1)-PC	0.026	—	—	0.077
(16:1)-G	43	13	74	8.5
(18:1)-G	37	24	27	3.5
(20:1)-G	23	12	10	1.8
(22:1)-G	24	13	7	1.1

ion-carrier complex from one interface to the other the reciprocal value  $1/k_{\text{off}}=3\mu\text{s}$  is the average time required for translocation. This time may be compared with the diffusion time  $\tau=d^2/2D$  of a spherical particle of the size of the carrier (radius  $\approx 0.7\text{ nm}$ ) across the same distance (membrane thickness  $d=5\text{ nm}$ ) in water (diffusion coefficient  $D=3\times 10^{-6}\text{ cm}^2\text{ s}^{-1}$ ) which is about  $0.04\mu\text{s}$ . From the rate constants, another important quantity may be calculated the maximum turnover rate  $f$  of the carrier.  $f$  is defined as the limiting transport rate which is approached under short-circuit conditions for infinite ion concentration on the cis-side and zero ion concentration on the trans-side.  $f$  may be calculated from the known values of the rate-constants (Langer 1972):

$$f = \left( \frac{1}{k_{\text{on}}} + \frac{1}{k_t} + \frac{1}{k_{\text{off}}} \right)^{-1} \quad (3)$$

In the above example,  $f$  is about  $3\times 10^4\text{ s}^{-1}$ . The high efficiency of valinomycin as an ion carrier mainly results from this high turnover rate whereas the binding constant for the ion  $k_{\text{on}}/k_{\text{off}}=1.5\text{ M}$  is rather low.

#### *Influence of membrane structure on the rate constant*

(a) *Variation of chain-length of the lipid* Carrier systems of the valinomycin type offer the possibility of studying the relationship between membrane structure and transport rates. The kinetics of valinomycin-mediated Rb<sup>+</sup> transport in membranes

made from different phosphatidylcholines (lecithins) has been studied with voltage jump experiments (Benz et al 1973). The phosphatidylcholines used in these experiments contained mono-unsaturated fatty acids of different chain-length ( $C_{16}$  to  $C_{24}$ ). It is seen from Table 1 that increasing the chain-length from  $C_{16}$  to  $C_{24}$  resulted in a drastic decrease of the association rate-constant  $k_a$  and the translocation rate constant  $k_t$  of free carrier (for technical reasons the two other rate constants could not be determined in all cases).  $k_a$  decreased by a factor of about 200 and  $k_t$  by a factor of about 20.

The decrease of the translocation rate constant  $k_t$  of the free carrier with chain length presumably reflects an increase of microviscosity of the membrane. For the surprisingly strong chain-length dependence of the association rate constant  $k_a$  at least two possible explanations have to be considered. One possibility consists in assuming that the uncomplexed carrier is present in the membrane in a conformational state which differs significantly from the compact conformation of the carrier in the complexed form. In this case complexation with the ion would require a major conformational change the rate of which should depend on the microviscosity of the environment. Another possibility is that the uncomplexed carrier is located more towards the hydrocarbon interior of the membrane. An ion entering the carrier from the aqueous solution then has to pass through the zone of the polar head-groups of the lipid. As the average location of the carrier with respect to the interface may depend

Table 2. Rate constants of valinomycin-mediated Rb<sup>+</sup> transport through membranes made from  $\alpha$ -monoglycerides (G) differing in the number of double-bonds in the fatty-acid residue: oleoyl (18:1) linoleoyl (18:2) linolenoyl (18:3)  $\Delta^5$ -eicosenoyl (20:1)  $\Delta^5$ -eicosadienoyl (20:2)  $\Delta^{5,8}$ -eicosatrienoyl (20:3) and arachidonoyl (20:4) (Ben, et al 1977)

The solvent for membrane formation was *n*-decane  $T=25^\circ\text{C}$

Lipid	$k_a/10^4 \text{ M}^{-1} \text{ s}^{-1}$	$k_b/10^4 \text{ s}^{-1}$	$k_{tr}/10^4 \text{ s}^{-1}$	$k_d/10^4 \text{ s}^{-1}$
(18:1)-G	37	24	27	3.5
(18:2)-G	67	13	143	6.2
(18:3)-G	74	8	50	9.6
(20:1)-G	23	12	10	1.8
(20:2)-G	34	9	39	3.2
(20:3)-G	38	5	136	9.4
(20:4)-G	42	3	240	12

on the nature of the hydrocarbon chain: a variation of chain length could result in a change of the energy barrier which has to be surmounted by the ion and therefore in a change of association rate. Possibly both effects contribute to the variation of  $k_a$  with lipid chain-length.

In a second series of experiments membranes made from monoglycerides differing in the fatty acid residue were studied (Benz et al 1977). In this case the variation of  $k_a$  with chain length was small, but again a pronounced decrease of  $k$  (also of  $k_{tr}$ ) with increasing chain length was found (Table 1).

(b) Variation of the number of double bonds. Kinetic parameters for valinomycin-mediated Rb<sup>+</sup> transport in membranes made from monoglycerides with varying degree of unsaturation (Benz et al 1977) are given in Table 2. Again  $k_a$  and  $k_b$  are not much sensitive to a variation in the number of  $m$  of double bonds. On the other hand the translocation rate constant  $k_{tr}$  (and to a lesser degree also  $k_d$ ) strongly increases with  $m$ : the change of  $k_{tr}$  being 4-fold between  $m=1$  and  $m=4$ . This variation of  $k_{tr}$  presumably results (at least in part) from an increased disorder and decreased microviscosity in membranes made from highly unsaturated monoglycerides. Another effect which may influence  $k_{tr}$  is the increase of dielectric constant of the membrane with increasing unsaturation. Such an increase of dielectric constant would reduce the energy barrier for the translocation of the charged complex.

(c) Effect of polar head-group. A comparison of the data for di-(16:1)-PC and (16:1)-G in Table 1 may serve to illustrate the effects of the polar head

group. Both lipids have identical fatty acid chains (palmitoleic acid) but differ in their polar residues. Dipalmitoleoyl phosphatidylcholine and glycerol monopalmitoleate differ considerably in all four rate constants. The most striking difference occurs in the value of the stability constant  $K_a = k_a/k_b$  of the complex,  $k_a$  being about 20 times larger in the case of the monoglyceride as compared with the phosphatidylcholine. Recently Hladky & Haydon (1973) have studied the stationary membrane conductance  $\lambda_a$  of lecithin and monoglyceride membranes in the presence of a cationic carrier system (nonactin/K<sup>+</sup>) and have presented arguments that the large difference in  $\lambda_a$  results from the existence of a dipolar potential in the membrane solution interface which makes the interior of a lecithin membrane more positive than the interior of a monoglyceride membrane. The finding that  $K_a$  is larger in a monoglyceride membrane than in a phosphatidylcholine membrane is consistent with this picture. However it is seen from Table 1 that also the two translocation rate constants  $k_{tr}$  and  $k_d$  differ considerably in membranes made from the two lipids. The influence on  $k_d$  may be discussed on the basis of a higher fluidity of the monoglyceride membranes compared with phosphatidylcholine membranes. Furthermore it is not clear to what extent a carrier can be influenced by an existing dipole potential. The valinomycin-cation complex has a diameter of 1.5 nm and a height of 1.2 nm: a molecule of this size necessarily creates a large local perturbation of the structure of the dipole layer.

(d) Incorporation of cholesterol. Incorporation of cholesterol into a glycerolmonoolate membrane

Table 3 *Effect of cholesterol on the rate constants of valinomycin-mediated Rb transport (Benz & Cros 1978)*

The membranes have been made from mixtures of glycerolmonooleate and cholesterol in *n*-hexadecane ( $T=25^{\circ}\text{C}$ )

The mole fraction of cholesterol (referred to total lipid) is given in the first column

$x$	$k_d/10^4 \text{ M}^{-1}$	$k_{tr}/10^4 \text{ s}$	$k_{cat}/10^4 \text{ s}$	$k_d/10^4 \text{ s}$
0	15	9	23	3.0
0.17	13	11	19	2.7
0.33	8.3	12	14	1.7
0.50	6.2	10	7.1	1.0
0.67	3.0	1	4.2	0.62
0.80	2.0	11	2.8	0.41

(Benz & Cros 1978) reduces  $k_d$ ,  $k_{cat}$  and  $k_b$  up to about 8-fold (Table 3). In these experiments *n*-hexadecane has been used as solvent for membrane formation. In this case the membrane thickness as determined by capacitance measurements is independent of cholesterol content (this is no longer true for membranes containing *n*-decane (Benz et al. 1977; Hanai et al. 1965)).

Cholesterol may affect the structure of the membrane and the kinetics of carrier transport in different ways (Hladky & Haydon 1973; Szabo 1974, 1976; McLaughlin 1976). By interacting with the hydrocarbon chains of the lipid molecules cholesterol may reduce the fluidity of the membrane and thereby decrease the translocation rate of the carrier. On the other hand there is experimental evidence that cholesterol changes the dipolar potential of the membrane so that the interior of the membrane becomes more positive (Hladky & Haydon 1973; Szabo 1974, 1976; McLaughlin 1976). Depending on the location of the adsorbed ion-carrier complex with respect to the plane of the dipoles a change in the dipolar potential may affect the stability constant  $K_a$  of the complex as well as the translocation rate constant  $k_{cat}$ . The observation that the effect of cholesterol on  $k_{cat}$  is about the same as on  $k_d$  seems to indicate that the translocation rate constant of the charged complex is not significantly influenced by the dipole potential. On the other hand it is seen from Table 3 that the stability constant  $K_a = k_d/k_b$  decreases about 7 fold if the mole fraction  $x$  of cholesterol is increased from 0 to 0.8. Both findings taken together may be interpreted by the assumption that the charged complex is located on the hydrocarbon side of the dipole layer. This interpretation has to be considered as tentative, however, in view of the difficulties

discussed above in the quantitative application of the dipolar-layer model to carrier mediated ion transport.

## REFERENCES

- ANDERSEN O. S., LEV A. A., FUCHS M., TING-BEALL, H. P. & TOSTESON D. C. 1977 Ion-transport across lipid bilayers mediated by protein valinomycin (PV). *Biophys J* 17, 211a.
- ANDREOLI T. E., TIEFENBERG M. & TOSTESON D. C. 1967 The effect of valinomycin on the ionic permeability of thin lipid membranes. *J Gen Physiol* 50, 257-2543.
- BENZ, R. 1978. Alkali-ion transport through lipid bilayer membranes mediated by amastin B and beauvericin. *J Membrane Biol* 43, 367-394.
- BENZ, R. & CROS D. 1978 Influence of sterol on ion transport through lipid bilayer membranes. *Biochim Biophys Acta* 506, 263-280.
- BENZ, R., FRÖHLICH O. & LAUGER, P. 1977 Influence of membrane structure on kinetics of carrier mediated ion transport through lipid membranes. *Biochim Biophys Acta* 46: 465-481.
- BENZ, R., GISEN H. F., TING-BEALL, H. P., TOSTESON D. C. & LAUGER, P. 1976 Mechanism of ion transport through lipid bilayer membranes mediated by peptide cyclo-(D-Val-L-Pro-L-Val-D-Pro). *Biochim Biophys Acta* 455, 665-684.
- BENZ, R. & LAUGER, P. 1976. Kinetic analysis of carrier-mediated ion transport by the charge-pulse technique. *J Membrane Biol* 77, 171-191.
- BENZ, R., STARK G., JANKO A. & LAUGER, P. 1973 Valinomycin-mediated ion transport through neutral lipid membranes: Influence of hydrocarbon chain length and temperature. *J Membrane Biol* 14, 339-364.
- EISENMAN G., CIANI S. M. & SZABO G. 1968 Some theoretically expected and experimentally observed properties of lipid bilayer membranes containing neutral molecular carriers of ions. *Fed Proc* 27, 1289-1304.



- HANAI T., HAYDON B. A. & TAYLOR J. 1965 The influence of lipid composition and of some adsorbed proteins on the capacitance of black hydrocarbon membranes. *J Theor Biol* 9 422-432.
- HLADKY S. B. & HAYDON D. A. 1973 Membrane conductance and surface potential. *Biochim Biophys Acta* 318 464-468.
- LEV A. A. & BUZHINSKY E. P. 1967 Cation specificity of the model bimolecular phospholipid membranes with incorporated valinomycin. *Citologiya* 9 102-105.
- LIBERMAN E. A. & TOPALY V. P. 1968. Selective transport of ions through bimolecular phospholipid membranes. *Biochim Biophys Acta* 163 125-136.
- LÄUGER, P. 1972. Carrier-mediated ion transport. *Science* 178: 24-30.
- MCLAUGHLIN S. 1976 Electrostatic potentials at membrane-solution interfaces. In: *Current topics in membranes and transport* (ed. F. Bronner and A. Kleinzeller), Vol 9. Academic Press, New York.
- MUELLER P. & RUDIN D. O. 1967 Development of K<sup>+</sup>-Na<sup>+</sup> discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem Biophys Res Commun* 26 398-404.
- OVCHINNIKOV YU. A. & IVANOV V. T. & SHKROB A. M. 1974 Membrane-active complexes. Elsevier Scientific Publishing Company, Amsterdam.
- STARK G. & BENZ, R. 1971 The transport of potassium through lipid bilayer membranes by the neutral carriers valinomycin and monactin. Experimental studies to a previously proposed model. *J Membrane Biol* 5 133-153.
- STARK G. BENZ, R. KETTERER, H. & LÄUGER, P. 1971 The rate constants of valinomycin-mediated ion transport through thin lipid membranes. *Biophys J* 11 981-994.
- SZABO G. 1974 Dual mechanism for the action of cholesterol on membrane permeability. *Nature* 252 47-49.
- SZABO G. 1976. The influence of dyotic potentials on the magnitude and the kinetics of ion transport in lipid bilayer membranes. In: *Extreme environments: Mechanisms of microbial adaptation* (ed. M. R. Helrich) pp. 321-348. Academic Press, New York.
- TING-BEALL, H. P. TOSTESON M. T. GISIN B. F. & TOSTESON D. C. 1974 Effect of peptide PV on the ionic permeability of lipid bilayer membranes. *J Gen Physiol* 63 497-508.





ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 482

THE EFFECTS OF ANTRAL  
DISTENSION ON GASTRIC ACID SECRETION  
AND ON THE RELEASE OF  
GASTROINTESTINAL HORMONES IN MAN

By

Ivi Mai Schöön

GÖTEBORG 1980



ACTA PHYSIOLOGICA SCANDINAVICA

SUPPLEMENTUM 482

From the Department of Surgery II Söhlgren's Hospital University  
of Göteborg Göteborg Sweden

THE EFFECTS OF ANTRAL DISTENSION ON GASTRIC ACID SECRETION AND  
ON GASTROINTESTINAL HORMONES IN MAN

by

Ivi-Mai Schöön

Göteborg 1980

This summary is based mainly on studies reported in the following papers:

- I Evidence for a defective inhibition of pentagastrin-stimulated gastric acid secretion by antral distension in the duodenal ulcer patient  
Schön I -H Berggårdh S Grützinger U & Olbe L  
Gastroenterology 75:363-367 1978
- II A study of the effect of antral distension on gastric acid secretion in man  
Schön I -H Lundqvist G Rehfeld J & Olbe L  
Digestion accepted for publ
- III The effects of beta adrenergic receptor blockade on the inhibition of gastric acid secretion and the release of pancreatic polypeptide by antral distension in healthy subjects  
Schön I -H Lundborg P Schwartz T W & Olbe L  
Scand J Gastroent accepted for publ
- IV The effect of antral distension in healthy subjects on betazole stimulated gastric acid secretion and the plasma concentration of immunoreactive neurotensin  
Schön I -H Bloom S R & Olbe L  
Scand J Gastroent accepted for publ
- V The effect of antral distension on the endocrine pancreas in man  
Schön I -H Sjöström L Holst J J Schwartz T W & Olbe L  
Scand J Gastroent accepted for publ

These papers are referred to in the text by their Roman numeral

## CONTENTS

### INTRODUCTION

Mechanical stimulation of gastric secretion  
Distension of the whole stomach in man  
Separate fundic and antral distension in man

### AIM OF THE PRESENT STUDIES

### METHODOLOGICAL CONSIDERATIONS

- A *Determination of gastric acid secretion*
- B *Antral balloon distension*

### RESULTS AND COMMENTS

#### *Separate balloon distension of the antrum*

- 1 as an inhibitor of pentagastrin-stimulated gastric acid secretion
  - a) in healthy subjects compared to duodenal ulcer patients
  - b) in an acid and a neutral intragastric milieu in healthy subjects with consideration of cimetidine as mediator
- 2 as an inhibitor of betazole (Histalog)-stimulated gastric acid secretion in healthy subjects with consideration of neurotensin as mediator
- 3 as an inhibitor of pentagastrin-stimulated gastric acid secretion in healthy subjects
  - a) with consideration of a possible adrenergic inhibitory reflex
  - b) with consideration of a possible dopaminergic inhibitory reflex
- 4 inhibiting release of gastrointestinal hormones partly by activating a gastro-pancreatic reflex to the endocrine pancreas

#### *Different patterns of gastric acid secretion in duodenal ulcer patients and healthy subjects*

### SUMMARY

### ACKNOWLEDGEMENTS

### REFERENCES



Distributors: Almqvist & Wiksell Tryckeri AB, Rindögatan 8  
S-751 81 Uppsala, Sweden

FROM THE DEPARTMENT OF SURGERY II, SAHLGREN'S HOSPITAL, UNIVERSITY OF GÖTEBORG, GÖTEBORG, SWEDEN

Abstract

*SCHÖN I -M. The effects of antral distension on gastric acid secretion and on the release of gastrointestinal hormones*

Antral distension in pouch dogs has been found to stimulate gastric acid secretion both via gastrin release and via reflex pathways to the parietal cell area. In an acid intragastric milieu, additional inhibitory action has been presented in the dog. In duodenal ulcer patients, graded balloon distension has been shown to stimulate gastric acid secretion without any release of gastrin and independent of intragastric pH. In healthy subjects, significant decreases of gastric acid secretion was evoked by antral distension. Gastric distension in man has resulted in stimulation of exocrine pancreatic secretion and release of pancreatic polypeptide. In the present study, antral distension by 150 cm<sup>3</sup> balloon volume had no effect on submaximal pentagastrin-stimulated gastric acid secretion in duodenal ulcer patients, whereas the secretion was inhibited by 20 % in healthy subjects ( $p < 0.05$ ). The inhibition of gastric acid secretion by antral distension persisted during neutralization of gastric contents and did not result in an increase of somatostatin concentration in portal venous blood or in gastric contents. Antral distension inhibited pentagastrin- and betazole-stimulated gastric acid secretion to the same extent, which excludes that the gastrointestinal peptides secretin, cholecystekinin and bombesin mediate the inhibition of gastric acid secretion. During antral distension, the concentration of neurotensin, potent inhibitor of gastric acid secretion, was unchanged in portal and peripheral venous blood in non-ulcer patients and in duodenal ulcer patients. Antral distension released pancreatic glucagon and pancreatic polypeptide and VIP to portal venous blood in both non-ulcer and duodenal ulcer patients, supporting the concept of gastro-pancreatic reflex to the endocrine part of pancreas. The results, however, indicate that no gastrointestinal peptide with established acid inhibitory property seems to mediate the inhibitory effect of antral distension on gastric acid secretion.

The rapid onset of the inhibition of gastric acid produced by antral distension and the immediate reversal of this inhibition mimics the pattern of reflex mechanism. The inhibitory effect of antral distension remained, however, after beta<sub>1</sub> and beta<sub>1,2</sub> receptor blockade and after intramuscular injection of the dopaminergic antagonist metoclopramide. An inhibitory reflex acting on ganglionic level is to some extent supported by preliminary data showing that antral distension in healthy subjects inhibited vagally mediated gastric acid secretion more effectively than pentagastrin-stimulated gastric acid secretion.

**Key-words:** antral distension, betazole, duodenal ulcer, gastric acid secretion, gastrin, gastro-pancreatic reflex, metoclopramide, metoprolol, pentagastrin, neurotensin, pancreatic glucagon, pancreatic polypeptide, propranolol, somatostatin.

"When you can measure what you are speaking about and express it in numbers you know something about it but when you cannot measure it when you cannot express it in numbers, your knowledge is of a vague and unsatisfactory kind"

Lord Kelvin

## Introduction

The study of gastric secretion is much younger than that of gastric motility the latter described as "coction" was already known to Hippocrates 460-370 B.C. The classical experiments of Reaumur (101) and Spallanzani (111) demonstrated that gastric digestion involved a chemical process. In 1833 Beaumont published his observations on a patient Alexis St. Martin who was left with a stomach fistula after a severe gunshot wound. By applying mechanical irritation such as the pressure of a thermometer to the internal coat of the stomach he observed that distinctly acid fluid was secreted (9). From this report Blondlot (16) and Bassow (6) conceived the idea of artificially producing gastric fistulae in dogs but they failed to excite gastric secretion by using mechanical irritation (friction). In these early investigations the site of stimulation (antral or fundic mucosa) is not distinctly described nor is the type of mechanical irritation. Mechanical scratching may have failed to stimulate acid secretion by damaging the mucosa. The introduction by Klemensiewicz (72) of the pouch method (in this instance in the pyloric region) suggested to Heidenhain the construction of a fundus pouch (55). Heidenhain appears to have been the first person to use balloon distension to stimulate gastric acid secretion in pouch dogs. His conclusion was that local stimulation had only local effect. Contradictory to this Pavlov (94) suggested that the mechanical stimulation of gastric acid secretion observed by earlier investigators may be of long reflex origin. Zelinoy and Sevitch working on dogs with pyloric pouches and gastric fistulae found that mechanical irritation of the pyloric mucosa augmented secretion in the fundus even after the "pyloric nerve" had been divided (136). In 1925 Lim Ivy and McCarthy reviewed the older experiments on the stimulation of gastric secretion by local (mechanical and chemical) stimulants. Lim et al performed their own investigations carried out in dogs with

some observations on normal man included (80) As far as mechanical stimulation was concerned they concluded that 1) "mechanical distension of the stomach stimulates gastric secretion in man and in the dog in the latter with or without intact vagus nerves" and 2) the stimulation of gastric secretion by mechanical distension is inhibited by the hypodermic administration of 1 mg atropine and by the introduction of fat into the small intestine In 1974 Debas and co-workers used a pouch dog very similar to that of Zelinoy and Savitch but they had totally different resources available to evaluate the results of antral distension (29) In their preparation of the innervated antral pouch gastric fistula and Heidenhain pouch distension of the antral pouch with sodium bicarbonate effectively stimulated the release of gastrin from the antrum and secretion of acid from both the gastric fistula and the Heidenhain pouch When the antrum was distended with acid both the serum gastrin and Heidenhain pouch responses were completely suppressed However the acid response from the body of the stomach with a gastric fistula was not abolished Total denervation of the antrum abolished the acid secretion response of the stomach body to distension of the antral pouch The conclusion was that antral distension evoked acid secretion by release of gastrin and by a vagal stimulatory pyloro-oxynitic reflex The effect of distension on antral G-cells appears to be mediated by intrinsic and vago-vagal reflexes (31) However the type of transmission in these reflexes is uncertain Small doses of atropine (less than  $25 \mu\text{g/kg}$ ) enhanced the gastrin response to sham feeding (39 113) insulin (37) and meal stimulation (128) in man Gastrin release evoked by bombesin was inhibited by cholinergic drugs in dogs (115) The results indicate: 1) cholinergic inhibition of gastrin release 2) reflex release of gastrin by a non-cholinergic other wise unknown transmission mechanism

In the experiments of Yanagashi and Debas on innervated antral pouch-dogs antral distension with acid resulted in the inhibition of pentagastrin-stimulated gastric acid secretion (135)

Distension of the fundic region of the stomach in the dog evokes gastric acid secretion apparently by stimulating a cholinergic reflex both of long vago-vagal and short intramural type (49

50 99 81) These reflexes have been characterized as oxynto-oxyntic (29) An oxynto-pyloric reflex pathway was suggested by experiments in which distension of a Pavlov pouch caused a release of gastrin from an innervated alkaline antral pouch (30)

In man the results of investigations on "mechanical stimulation" of gastric acid secretion have revealed some important differences compared to the results in dogs

In 1901 Schöle failed to stimulate gastric acid secretion by mechanical stimulation in man using vigorous touching of the stomach wall with a soft rubber tube (106) Lin and co-workers performed whole stomach distension in man with rubber balloons (80) and three out of five men showed a definite stimulation of gastric acid secretion By distending the stomach of healthy subjects with 500 ml of a chemically inert test meal at pH 5.0 in healthy subjects Richardson and co-workers were able to conclude that distension is an important stimulant of the acid-secretory response to a meal and that stimulation was not mediated by gastrin release (102) Schrupf and Stedens (105) in their studies of balloon distension of the whole stomach in healthy subjects also failed to evoke any increase of plasma gastrin levels The same results were obtained by Soares et al (110)

Separate fundic distension in healthy subjects as well as in duodenal ulcer patients resulted in an increased gastric acid secretion with increasing distension volume up to 600-800 ml (51) The highest acid output amounted to just above 50 per cent of the maximal secretory response to pentagastrin The stimulation by fundic distension was blocked by 1 v. tropine and partially inhibited by proximal gastric vagotomy (52) Fundic distension was thus considered to stimulate gastric acid secretion by means of an atropine-sensitive reflex mechanism involving both short intramural and long vago-vagal pathways Plasma gastrin levels were unchanged during separate fundic distension in duodenal ulcer patients (52) even when gastric contents were neutralized

Previous studies have shown evidence that antral distension

healthy subjects does not increase basal acid secretion irrespective of whether the pH of the gastric contents is low (about 2.5) or high (above 7) (12, 10). In fact a slight but statistically significant decrease of acid secretion was observed in healthy subjects. Similar studies in duodenal patients, however, show evidence that separate graded antral distension increases acid secretion in a stepwise fashion. At 150 cm<sup>3</sup> balloon volume the secretory response amounted to about 35 % of the maximal acid response to pentagastrin (12, 11). Basal plasma gastrin level remained unchanged by antral distension in these duodenal ulcer patients even when the intragastric milieu was alkaline (11). The acid response in duodenal ulcer patients was suggested to be mediated via a pyloro-duodenal reflex (11) quite opposite to the findings in dogs where the gastrin mechanism predominated (29). A marked difference in the acid responses to the antral distension between duodenal ulcer patients and healthy subjects with stimulation occurring only in the ulcer patients was demonstrated (12).

Apparently antral distension in man affects gastric acid secretion in two ways: one stimulating and the other inhibiting gastric acid secretion. As regards the stimulatory effect, this has been suggested to be mediated by means of a reflex activation. As far as the inhibitory response is concerned, the mechanism involved is obscure. Previous studies show evidence that there are probably several nervous and/or neurohormonal factors which might directly or indirectly suppress gastric secretion. For example, vagal stimulation has been shown to result in increase of the gastric HCO<sub>3</sub> secretion (40) and in acid intragastric milieu such stimulation releases somatostatin known to inhibit gastric secretion (122, 53). Moreover, there is strong evidence that antral distension directly activates adrenergic neurones suppressing gastric motility (1) and that gastric distension might also stimulate the gastro-pancreatic reflex to the exocrine part of the pancreas shown to involve both vagal and splanchnic nerves (130, 132). Antral distension has recently been shown to activate gastric receptors with slowly adapting charge recorded in splanchnic nerve afferents (41). Both basal and pentagastrin-stimulated gastric acid secretion is inhibited by adrenergic agonists (28, 45) without decreasing gastric blood flow.

Adrenergic presynaptic inhibitory effects on gastrointestinal motility have also been demonstrated (125). It therefore appears possible that adrenergic reflex mechanisms evoked by antral distension might be involved in inhibition of gastric acid secretion.

As far as the gastro-pancreatic reflex pathway is concerned the reflex to the exocrine part of pancreas has been established both in the dog and in man (14, 82, 131, 129, 130, 132). Vagal reflex release of pancreatic polypeptide available from both exocrine and endocrine part of the pancreas (76) has been strongly suggested in man (107). However, since the amount of this hormone as released by sham feeding is diminished and the response delayed after propranolol treatment a beta adrenergic component was indicated (3). A gastro-pancreatic reflex release of pancreatic glucagon, a potent inhibitor of gastric acid secretion, also known to be released by both cholinergic and adrenergic stimulation (34) seems to be a neurohumoral possibility of inhibition of gastric acid secretion.

Also a dopaminergic reflex inhibition may be considered because intravenous infusion of dopamine inhibits both basal and pentagastrin-stimulated gastric acid secretion in man in dog (123, 24, 124) and this inhibition is abolished by pretreatment with metoclopramide (120).

Besides the above-mentioned neurohormonal release purely hormonal inhibition of gastric acid secretion also has to be taken into account. Thus secretin, cholecystikinin, bulbogastrone and probably also neurotensin inhibit gastric acid secretion when stimulated by pentagastrin. However, these hormones fail to inhibit acid secretion when histamine is used for stimulation (47, 44, 67, 57, 118, 2, 22). Although the failure of antral distension to inhibit betazole-stimulated gastric acid secretion can reasonably exclude these gastrointestinal hormones as possible mediators of the antral-distension-released gastric-acid inhibition the demonstration of unchanged blood concentration of the individual hormones during antral distension will support such a statement.

## AIM OF THE PRESENT STUDIES

The central and local nervous and neurohormonal control of gastric acid secretion is in many respects still poorly understood. Previous results on vagal stimulation and/or hormonal inhibition of gastric acid secretion are to a considerable extent controversial. The divergent opinions met in the literature may often be explained by different experimental conditions, incomparable patients or species examined and the use of unphysiological stimuli. Moreover not until recently methods have become available to study the gastrointestinal hormones by determination of the fluctuations in their blood concentrations in response to different physiological stimulatory/inhibitory effects.

The principal aim of the present study was therefore:

- 1 To compare qualitatively and quantitatively the response of separate antral balloon distension on pentagastrin-stimulated gastric acid secretion in duodenal ulcer patients and healthy subjects. To explore more in detail the mechanisms that might be involved in these responses particularly as regards nervous and neurohormonal mechanisms.
- 2 To study qualitatively and quantitatively the response of separate antral balloon distension on betazole stimulated gastric acid secretion in healthy subjects. This investigation was considered of particular interest since inhibition of betazole stimulated gastric acid secretion by antral distension would exclude secretin, cholecystokinin and bulbogastrone as possible mediators.
- 3 To determine the plasma concentration of somatostatin, neurotensin and glucagon, all potent inhibitors of gastric acid secretion, in portal and peripheral venous blood and if possible also to analyse somatostatin in gastric contents. In this context it was considered of interest also to assess the gastrin concentration in portal and peripheral venous blood for further evaluation of a possible release of somatostatin which should suppress the gastrin concentrations (122-53).
- 4 To evaluate whether  $\beta$ -adrenergic reflex activation or a dopaminergic transmission might be a cause of gastric acid inhibition as evoked by antral distension.
- 5 To study to what extent separate antral balloon distension

in healthy subjects might affect the endocrine part of the pancreas and the relationship of pancreatic glucagon, insulin and glucose in peripheral venous blood in an attempt to shed further light on the gastro-pancreatic reflex mechanisms.

## METHODOLOGICAL CONSIDERATIONS

### *A. Determination of gastric acid secretion*

To check recovery, constant perfusion of the stomach using 225 ml  $(15 \text{ min})^{-1}$  of a marker solution (8 mg phenol red per litre) water was employed. The nasogastric perfusion tube (Intramedic PE 160) was inserted into the stomach with its tip 5 to 10 cm below the cardia. A high perfusion volume facilitated mixing of the marker and the gastric juice. The gastric contents were continuously aspirated using a suction pump with intermittent negative pressure ( $\sim 50 \text{ mmHg}$  once/second). Due to continuous aspiration gastric distension was avoided. It might be argued that the marker might to some extent be absorbed interfering with the quantitative estimations. However, such an error appears less likely. The loss of phenol red through the canine gastric mucosa in a Heidenhain pouch during histamine stimulated secretion is in fact less than 5 per cent (17) and the absorption of phenol red in the lumen of the human stomach is probably still lower and independent of the pH of the gastric contents (58, 64, 95). Moreover, the phenol red concentration in 10 ml of each sample was determined spectrophotometrically at a wavelength of 565  $\mu\text{m}$  after filtration (Millipore filter 1.2  $\mu\text{m}$ ) and alkalization with 0.04 ml of 2.4 moles per litre of NaOH, making any interference with various bile pigments minimal. Assuming similar percentage loss of gastric acid and phenol red via the pylorus, the total amount of secreted acid could be calculated from the recovered quantity of phenol red. Quantitative estimation of duodeno-gastric reflux was not used in the present studies.

Determination of the concentration of hydrogen ions was carried out by potentiometrical titration of 100 ml of the sample to pH 7.0 when water perfusion of the stomach was used. In the experiments with alkaline buffer perfusion of the stomach (II) the perfusate consisted of phenol red in 1/15 M Sørensen's phosphate



buffer pH 8.3 (162 mosm  $l^{-1}$ ) the perfusion rate still being 225 ml  $(15 \text{ min})^{-1}$ . 100 ml of each sample was titrated to pH 9.5. The titration value of the sample minus the titration value of the infused amount of buffer solution per 15 min (titrated to pH 9.5) was expressed as acid output in  $\text{mmol } (15 \text{ min})^{-1}$ .

## B. Antral balloon distension

A small thin-walled latex balloon firmly tied to a polyethylene tube of 3 mm diameter that was attached to a double lumen nasogastric tube (Salem sump no 14) in such a way that the latex balloon when insufflated was placed in front of the tip of the sump tube. Loosely attached to these two tubes was a third thin polyethylene tube (for perfusion of the water or buffer solution of phenol red) which could be retracted with its tip placed in the duodenal region of the stomach.

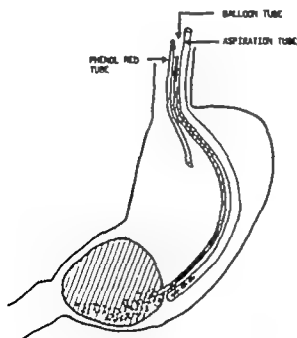


Fig 1 Schematic illustration of the arrangement of the nasogastric tubes

With the empty balloon wrinkled around the Salem sump tube the equipment could be passed through a nostril and placed in the stomach. The position of the nasogastric tube was controlled by fluoroscopy of the X ray visible thread in the Salem tube. After the determination of basal acid secretion during three to four 15 min periods the balloon was filled with 150 cm<sup>3</sup> water solution of a roentgen contrast medium (Gastrografin) (Fig 1). The intraballoon pressure was calculated to about 32 cm H<sub>2</sub>O. The position of the balloon was checked throughout the experiment by fluoroscopy. The volume of 150 cm<sup>3</sup> was chosen to ensure a separate antral distension because at balloon volumes exceeding 150 cm<sup>3</sup> the balloon tended to bulge into the corpus region (12).

For anatomical reasons and to prevent sliding of the balloon in the laparotomy experiments this was filled with 150 cm<sup>3</sup> air. The position of the balloon was then checked manually throughout the 30 min period of distension and in all experiments the volume of contrast or air was carefully measured at deflation of the balloon. The distension of the antrum using closed balloon system is considered to be predominantly isometric. The balloon remained in place in the antrum as checked by fluoroscopy and no discomfort was observed in any patient during its distension.

The advantage of balloon distension as compared with the more conventional fluid distension are obvious: no chemical or osmotic influence on the distended area can interfere with mechanical stimulation. The antrum has a complex autonomic innervation. Most of the abdominal vagus and the splanchnic nerves consist of afferent nerve fibres from the gastro-intestinal tract. Single fibre records from vagal afferents (93, 60, 61) reveal two distinct categories of gastric mechanoreceptors: slowly adapting mechanoreceptors and rapidly adapting mechanoreceptors with some chemoreceptor properties (62) and a sustained response is obtained on a steady distension (93, 60). Moreover, passive distension of an visceral organ and isometric contraction in the receptor area has been shown to activate slowly adapting mechanoreceptors which are considered to be functionally coupled in series (78). Gastric receptors with splanchnic nerve afferents give slowly adapting discharge to antral distension (41).

similar to the impulses in splanchnic nerve afferents from os chemoreceptors in the mesentery (91) There is evidence that entral distension in the cat elicits two different gastro-gastric reflex mechanisms suppressing the gastric smooth muscle activity These reflexes are mediated via the vagal and splanchnic nerves (1)

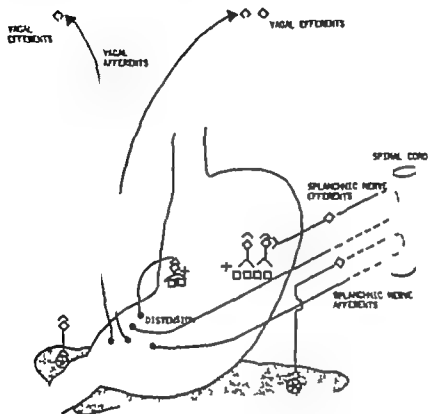


Fig 2 Schematic diagram of possible Vagal and Splanchnic reflex mechanisms

As shown in Fig 2 it appears that entral distension as applied in the present study could possibly activate several afferent neurones eliciting at least theoretically stimulatory and/or inhibitory effects on gastric acid secretion Thus equivocal and divergent results often obtained in previous studies of gastric function may partly be explained by the fact that different modes of mechanical stimulation have often been utilized

## RESULTS AND COMMENTS

1a Separate balloon distension resulted in a 20 % inhibition of submaximal pentagastrin-stimulated gastric acid secretion in 6 healthy subjects and produced no inhibition at all in 6 duodenal ulcer patients (I). Inhibition was a constant phenomenon in 30 healthy subjects (II) with an increase of the pH of the gastric contents during the distension period. The mean output volume significantly decreased during the distension period with an almost complete recovery of phenal red (mean 92.96 %) throughout the experiments. This excludes the possibility that the decrease in acid response was due to an increased alkaline reflux or an increase of alkaline secretion (40) in the stomach.

The result suggests that antral distension elicits stimulatory as well as inhibitory mechanisms affecting gastric acid secretion in man. The inhibitory mechanism seems to be defective in duodenal ulcer patients. The inhibitory mechanism appears to predominate the balance in healthy subjects and thus the stimulatory mechanism is only detectable in duodenal ulcer patients. In patients with an active duodenal ulcer the acid response to separate antral distension amounted to about 35 % of the peak acid output after pentagastrin stimulation (12).

1b Neutralisation of the intragastric milieu with a pH increase from a mean of 7 to 6.8 during the distension period did not change the inhibitory effect of antral distension on pentagastrin-stimulated gastric acid secretion in six healthy subjects (II). Vagal stimulation produces a release of somatostatin in an acid intragastric milieu in the cat (122) and in the pig (53). Somatostatin inhibits gastric acid secretion and gastrin release in the dog (5) and in man (97, 100). In the dog an increase of the plasma concentration of gastrin has been observed during antral distension with an alkaline intragastric milieu but no increase occurred with an acid intragastric milieu (29). In man no increase of the plasma concentration of gastrin has been observed during antral distension (11) irrespective of the pH of the intragastric milieu (10).

In the present study no change in the plasma concentration of

portal venous blood was observed in duodenal ulcer patients nor in non-ulcer patients (II). This strengthens the opinion that antral distension in man does not stimulate gastrin release. In the present investigation (II) no change in the somatostatin concentration of portal venous blood was observed in non-ulcer patients nor was there any release of somatostatin into the gastric lumen in healthy subjects. Unchanged plasma somatostatin and gastrin concentrations during antral distension indicate that this inhibition is not mediated via somatostatin (II). The inhibition of gastric acid secretion evoked by antral distension which persisted during perfusion of the stomach with alkaline buffer also indicate that this inhibition is not mediated by somatostatin (II).

2. Antral distension resulted in a 20 % inhibition of gastric acid secretion stimulated by a continuous intravenous infusion of either betazole (Histalog)  $10 \text{ mg kg b.w.}^{-1} \text{ h}^{-1}$  or pentagastrin (IV). The plasma concentration of neurotensin in portal blood did not change in non-ulcer patients nor in duodenal ulcer patients during antral distension (IV).

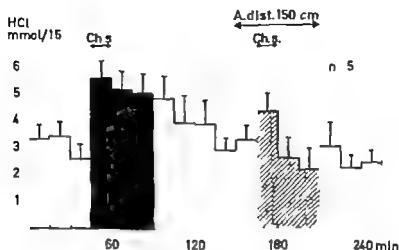
The gastrointestinal hormones secretin, cholecystokinin and bombesin are known to inhibit pentagastrin- but not histamine stimulated gastric acid secretion (69-68). Thus the consistent inhibition of Histalog-stimulated gastric acid secretion by antral distension indicates that it cannot be mediated by these gastrointestinal peptide hormones (IV).

The intravenous infusion of synthetic neurotensin inhibited pentagastrin-stimulated gastric acid secretion in dogs (2) to approximately 50 %. Therefore the concentration of immunoreactive neurotensin was determined during antral distension (IV). The plasma concentration of neurotensin remained unchanged and thus neurotensin could be excluded as a possible mediator of gastric acid inhibition produced by antral distension.

Vasoactive intestinal polypeptide (VIP) is known to inhibit both pentagastrin- and betazole stimulated gastric acid secretion (83-84). VIP could hypothetically be the mediator released by antral distension (36-25). Preliminary unpublished data has

shown a slight increase in the plasma concentration of VIP in portal blood during entral distension without any difference between duodenal ulcer patients and non-ulcer patients

a)



b)

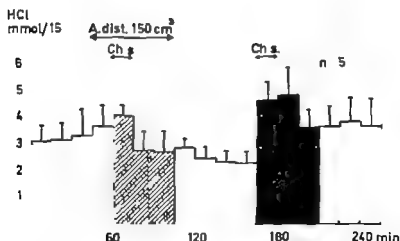


Fig 3 The effect of entral distension on gastric acid secretion stimulated by modified sham feeding. Mean  $\pm$  S.E. 5 healthy subjects. The hatched areas have been compared.

a) The control test precedes the experiment

b) The experiment precedes the control test

Ja The rapid onset of the inhibition of gastric acid secretion produced by antral distension and the immediate reversal of this inhibition mimicks the pattern of a reflex mechanism. Moreover some further support to the inhibitory reflex theory is delivered by the 30 % / 42 % inhibition of vagally stimulated gastric acid secretion in 5 healthy subjects (Fig 3a and b) where as humorally stimulated gastric acid secretion in healthy subjects was inhibited to 20 % by antral distension (I-IV)

Adrenergic reflex inhibition is an additional conceivable explanation. Earlier investigations in dogs described a hyperchlorhydria after bilateral splanchnicectomy (89-92) and an increase in the occurrence of peptic ulcer after coeliac ganglionectomy (79). Sympathectomy in rats increased gastric acid secretion and the incidence of experimental peptic ulceration (90). Adrenergic transmitter antagonists stimulated gastric acid secretion (65-74). Beta adrenergic agonists have produced a marked inhibitory effect on gastric acid secretion (119-28-45). In a majority of the investigations it has not been possible to differentiate a direct adrenergic inhibition from an inhibitory effect secondary to diminished mucosal blood flow. Some beta adrenergic agonists however inhibit gastric acid secretion without influence on the blood flow (28). In the present study neither beta<sub>2</sub> nor beta<sub>1+2</sub> adrenergic receptor blockade (III) changed the inhibition of pentagastrin-stimulated gastric acid secretion evoked by antral distension. This suggests that the inhibitory effect of antral distension on gastric acid secretion is not mediated via a beta-adrenergic reflex. However the results do not exclude the possibility of an adrenergic inhibitory reflex mechanism. Adrenergic inhibition on a ganglionic level by activation of alpha adrenergic receptors (125-133) may still be conceivable.

3b The inhibition of basal and pentagastrin-stimulated gastric acid secretion induced by dopamine was abolished by an i.v. injection of 10 mg of metoclopramide in healthy subjects (24). This indicates a dopaminergic inhibitory effect which can be blocked by metoclopramide. However a metoclopramide dose of 10, 20 or 40 mg given intravenously had no effect on basal acid secretion or on antral histamine-stimulated gastric acid

secretion in healthy subjects or duodenal ulcer patients before and after truncal vagotomy (88 87)

In the present investigation (IV) pretreatment with 10 mg of metoclopramide did not influence the inhibition of pentagastrin-stimulated gastric acid secretion induced by entral distension in 7 healthy subjects. The results do not support the view that a dopaminergic mechanism acts as mediator for the inhibitory effect evoked by entral distension (IV)

4 Distension of the innervated stomach has been shown to stimulate pancreatic exocrine secretion (32 82 131 129 132 54 15). This response has proved to be dependent upon both vagi and splanchnics (130 132). The pancreas receives a mixed autonomic innervation involving at least cholinergic and adrenergic components (121 56) to both the exocrine and endocrine part of the pancreas. Pancreatic glucagon is released by both cholinergic and adrenergic nerve stimulation (34). Insulin is released by cholinergic stimulation (98) and pancreatic polypeptide vi predominantly vagal stimulation (109 114 108). A gastropancreatic reflex to the endocrine part of pancreas may exist.

Separate fundic and entral distension in man elicited an increase in the secretion of pancreatic polypeptide (107). The cells secreting pancreatic polypeptide are present both in the pancreas and in the gut (76). The endocrine part of pancreas is responsible for the release of pancreatic glucagon. In the present study (V) the concentration of pancreatic glucagon in portal venous blood increased during entral distension.

Proximal gastric vagotomy abolished the release of pancreatic polypeptide evoked by separate fundic distension (107). This supports the existence of a vagally mediated gastro-pancreatic reflex to the endocrine part of pancreas.

In the present study a marked increase in the plasma pancreatic polypeptide concentration occurred in portal and peripheral venous blood following entral distension both in duodenal ulcer patients and in patients undergoing cholecystectomy (V). The concentration of pancreatic glucagon in portal venous blood



increased during entral distension in 15 patients (8 cholelithiasis patients and seven duodenal ulcer patients) (V). Neither the release of pancreatic polypeptide nor pancreatic glucagon can explain the inhibition of gastric acid secretion.

The increase in concentration of pancreatic polypeptide in peripheral venous blood during entral distension was slightly suppressed by a  $\beta_{1+2}$ -adrenergic antagonist. A similar suppression did not occur after medication with  $\beta_1$  adrenergic antagonist (III). This indicates the possible involvement of a  $\beta_2$  adrenergic component in the release of pancreatic polypeptide.

Separate entral distension in healthy subjects evoked a significant increase in the plasma concentration of motilin both with and without a background infusion of pentagastrin (unpublished observations). Motilin-secreting cells are located predominantly in the jejunal part of the small intestine (19). This indicates that entral distension also influences the endocrine part of the jejunum by a possible gastrojejunal reflex?

Distension or mechanical stimulation of different parts of the gastrointestinal tract activates complex and differentiated mechanisms that are briefly outlined in Table I. It appears that the functional involvement of the extrinsic and intrinsic control of the gastrointestinal tract in general differs considerably in its various compartments. Nevertheless great similarities still exist and this is particularly so as regards the gastric and colorectal section. Thus entral distension like distension or mechanical stimulation of the rectum elicits a complex response pattern activating both smooth muscles and the secretory glands. Beside short or long nervous reflex activation a direct or indirect hormonal release appears to play an important part in these response patterns. It appears likely that these reactions to distension (on meals or at defecation) are physiological and constitute well-graded reflex adjustments of the respective organ functions.

Tabl. I

## SOME EFFECTS BY DISTENSION OF DIFFERENT PARTS OF GASTROINTESTINAL TRACT

DISTENSION OF	STIMULATORY EFFECT	RELAXATORY/INHIBITORY EFFECT	RELEASE OF GASTRO-INTESTINAL PEPTIDES
Distal oesophagus		Vago-vagal non-adrenergic receptor relaxation (86 66 1)	VIP (36)
Gastric fundus	Gastric acid secretion (91)		Gastrin in dog (30)
	Exocrine pancreatic secretion probably partly adrenergic (130)		Vagal pancreatic polypeptide (107)
Gastric antrum	Gastric acid secretion in duodenal ulcer patients (12)	Inhibition of gastric acid secretion in healthy subjects (1)	Gastrin in dog (29)
			Pancreatic polypeptide release probably partly beta <sub>2</sub> -adrenergic (III)
			Pancreatic glucagon (V)
			VIP Motilin (unpublished)
		Vago-vagal non-adrenergic reflex relaxation (86 66 1)	
Small intestine	Contraction oral to stimulus (7)	Relaxation aboral to stimulus (7)	Secretorin (23 43)
			VIP (36)
		Intestino-intestinal inhibition (73)	
		Vasodilatation (13)	
Colon	Contraction oral to stimulus (8)	Relaxation aboral to stimulus (8)	Secretorin (77)
		Vasodilatation (intracolo) (38)	VIP (36)
		Colo-colonus inhibition (73)	
		Vasodilatation (pelvopelvic) (38)	
Rectum	Pelvic-pelvic and colonic contraction and secretion (39)	Vasodilatation (39)	
		Anal relaxation (46)	

## *Different patterns of gastric acid secretion in duodenal ulcer patients and healthy subjects*

A considerable number of differences in gastric secretory pattern have been established between duodenal ulcer patients and healthy subjects. The cause of these differences is unknown as is the importance of these differences for the pathogenesis of duodenal ulcer disease (104). It has been suggested that certain patients illustrate a partial abnormality of gastric secretion (104). Duodenal ulcer patients have been found to have a significantly higher maximal acid response to pentagastrin (70) histamine (442) Histalog (134) and meal stimulation (42). However, in one study (85) no difference was found in the peak rate of acid secretion in response to meal stimulation between duodenal ulcer patients and healthy subjects, but the secretory response was prolonged in duodenal ulcer patients. The increased capacity to secrete acid has been related to the greater number of parietal cells in duodenal ulcer patients (27), the cause of which is completely unknown.

Duodenal ulcer patients display a greater increase in gastrin concentrations after meal stimulation (124) or an intravenous infusion of isoprenaline (20) or adrenalin (21) than healthy subjects. It has been suggested that there exists a G-cell hyperplasia in duodenal ulcer patients compared to healthy subjects (27). However, the amount of antral and duodenal gastrin determined by biological methods (35) did not differ between duodenal ulcer patients and non-ulcer subjects.

Another difference is the increased sensitivity for pentagastrin stimulation that has been observed in duodenal ulcer patients (63, 96, 103, 116). A higher sensitivity for pentagastrin in duodenal ulcer patients might be in keeping with an increased vagal tone (33). Indirect evidence speaks against vagal hyperactivity, however, since the frequency of high basal plasma concentrations of pancreatic polypeptide is the same in duodenal ulcer patients as in healthy subjects (109). Pancreatic polypeptide is mainly released by vagal stimulation.

Another conceivable explanation of the increased sensitivity

of duodenal ulcer patients to hormonal stimulation are defective inhibitory mechanisms. Several defective inhibitory mechanisms have been established. Acidification of the gastric contents has been found to reduce the inhibition of acid secretion stimulated by a meal (127) or by insulin hypoglycaemia (112) in duodenal ulcer patients compared to healthy subjects. However defective acid inhibition of the gastric secretory response to a meal does not seem to be a constant finding in duodenal ulcer patients (117). Fat in the duodenum inhibited meal stimulated gastric acid secretion to an equal extent in duodenal ulcer patient as in healthy subjects. However gastrin release was suppressed only in healthy subject (41). Submaximal pentagastrin-stimulated gastrin acid secretion was significantly less inhibited in duodenal ulcer patients compared to healthy subjects when small volumes (20 ml) or oleic acid were infused into the duodenum (71).

The present study has established another abnormality in the acid secretory pattern of duodenal ulcer patients i.e. the defective inhibition of acid secretion by antral distension in duodenal ulcer patients (1). Antral distension offers certain advantages in elucidating abnormalities of the secretory pattern of duodenal ulcer patients. The experimental model is simple and the inhibitory effect is absent in duodenal ulcer patients but is constantly observed in healthy subjects. Gastrointestinal peptides with properties that inhibit gastric acid secretion do not seem to mediate the inhibitory effect of antral distension. The data obtained complies with a non-beta adrenergic non-dopaminergic reflex inhibitory mechanism possibly acting on a ganglionic level.

#### SUMMARY

Balloon distension of the antrum in healthy subjects decreased basal acid secretion and consequently resulted in a 20% inhibition of pentagastrin-stimulated gastric acid secretion independent of the intragastric pH. Antral distension stimulated gastric acid secretion in duodenal ulcer patients and did not evoke any change of pentagastrin-stimulated acid secretion. The plasma concentration of gastrin in portal and peripheral venous blood

did not change in duodenal ulcer patients nor in non-ulcer patients during entral distension. Thus entral distension evoked both an inhibitory and a stimulatory effect on gastric acid secretion in man. The inhibitory mechanism predominated in healthy subjects and was defective in duodenal ulcer patients. The stimulatory mechanism in duodenal ulcer patients is probably a reflex activation and gastrin release from the antrum does not participate.

The nature of the inhibitory mechanism was investigated in healthy subjects. Entral distension inhibited pentagastrin- and Histalog-stimulated gastric acid secretion to the same extent. This excludes gastrointestinal peptides such as secretin, cholecystokinin and bulbogastrone as possible mediators of the inhibition of gastric acid secretion.

During entral distension the concentrations of somatostatin and neurotensin were unchanged in portal and peripheral venous blood. Somatostatin was undetectable in the gastric contents. Entral distension released pancreatic glucagon and pancreatic polypeptide into portal venous blood in amounts which are probably insufficient to inhibit gastric acid secretion. Pancreatic glucagon, pancreatic polypeptide and vasoactive intestinal polypeptide (VIP) were released in similar amounts by both duodenal ulcer and non-ulcer patients. The results indicate that no gastrointestinal peptide with an established acid inhibitory property mediated the inhibitory effect of entral distension on gastric acid secretion in healthy subjects. The results support however the concept of gastro-pancreatic reflex to the endocrine part of the pancreas with release of at least pancreatic polypeptide and pancreatic glucagon.

The rapid onset of the inhibition of gastric acid secretion produced by entral distension and the immediate reversal of this inhibition mimicks the pattern of a reflex mechanism. The inhibitory effect of entral distension remained however after  $\beta_{1}$  and  $\beta_{1+2}$  receptor blockade and after an intramucosal injection of the dopaminergic antagonist metoclopramide. An inhibitory reflex acting at the ganglionic level is supported to some extent by preliminary data showing that entral distension

inhibited vagally stimulated gastric acid secretion more efficiently than pentagastrin-stimulated gastric acid secretion in healthy subjects

#### Acknowledgements

*I wish to express my sincere gratitude*

to Professor Ragnar Romanus who permitted the commencement of this thesis and who has followed it with kind support and interest

to Professor Nils E. Kock head of the Department of Surgery II Sahlgren's Hospital during the greater part of my work on this thesis for his invaluable assistance throughout this study

to associate Professor Lars Olbe for the eminent way in which he introduced me to the field of gastric secretory physiology and for his invaluable support and guidance throughout the work

to associate Professor Leif Hultén for valuable discussions

to my colleagues and co-authors Stig Bergegårdh and Ulrich Grötzingar for valuable discussions and to my co-authors Stephen R. Bloom, Jan Fehrenkrug, Jens Holst, Per Lundborg, Gudmar Lundqvist, Jens Rehfeld, Thue Schwartz and Lars Sjöström (in alphabetical order) who have shown keen interest in the study and who have encouraged me in the correspondence

to my colleague Ian Milson for his inestimable assistance in revising the English

to Eva Albræksten, Barbro Nyman, Annerita Ioving, Margareta Uglend, Margareta Öhman and Gunilla Eriksson for their excellent technical assistance at all times throughout the different studies

to Hugo Svensson who has skillfully drawn all the figures subsequently photographed by Erika Pfeiffer

to miss Siv Josefsson for skilful typing of the manuscripts

to the volunteers and patients for their invaluable patience throughout the experiments. Without their assistance this study would never have been completed.

I also wish to express my gratitude to my husband Nils Horman for his constant invaluable support.

Grants from the Swedish Medical Research Council (project 17 X-760) the Danish Medical Research Council (512 10227) Göteborgs Läkaresällskap Linder's Fond and Harald Jeansson's Stiftelse have supported this work.

AB Hålsjö Hbindal - Sweden - generously covered part of the expenses for the translation of the manuscripts.

References

- 1 Abrahamsson H. Studies on the inhibitory nervous control of gastric motility. *Acta Physiol Scand* suppl 390 1973
- 2 Andersson E, Chang D, Folkers K and Rosell S. Inhibition of gastric acid secretion in dogs by Neurotensin. *Life Sciences* 19:367-370 1976
- 3 Arnold R, Koop H, Ebert R, Becker H D and Creutzfeldt W. Effect of atropine, propranolol and cisotidine on serum pancreatic polypeptide (PP) and GIP during sham feeding in man. Abstr. 13th Annual Meeting of European Society for Clinical Investigation 22-24 March 1979, p. 2. Published by INSERM France
- 4 Baron J H. An assessment of the augmented histamine test in the diagnosis of peptic ulcer. Correlations between gastric secretion, age and sex of patients, and site and nature of the ulcer. *Gut* 4:243-253 1963
- 5 Barrow D, de A A J, Bloom S H and Baron J H. Direct inhibition of gastric acid by growth-hormone release inhibitory hormone in dog. *Lancet* I:886-889 1975
- 6 Bassov. *Bull soc natur de Moscou* 1843 XVI:351 (quoted from Babkin H T, *Secretory mechanisms of Digestive Glands* New York ed Hoeber 1950)
- 7 Bayliss W H and Starling E H. The movements and innervation of the small intestine. *J Physiol* 24:99-143 1899
- 8 Bayliss W H and Starling E H. The movements and the innervation of the large intestine. *J Physiol*, 26:107-118 1900-1901
- 9 Beaumont W. Experiments and observations on the gastric juice and the physiology of digestion. Plattsburg, New York ed F P Allen 1833
- 10 Berggårdh E, Knutson U and Olbe L. The effect of intra-gastric neutralization on the gastric acid response to enteral distension in man. *Scand J Gastroent.* 10:625-631 1975
- 11 Berggårdh E, Nilsson G and Olbe L. The effects of enteral distension on acid secretion and plasma gastrin in duodenal ulcer patients. *Scand J Gastroent.* 11:475-479 1976
- 12 Berggårdh S and Olbe L. Gastric acid response to entrum distension in man. *Scand J Gastroent.* 10:171-176 1975



- 13 Biber H Vasodilator mechanisms in the small intestine  
*Acta Physiol Scand* suppl 401 1973
- 14 Bizard G and Boulet L Influence de la distension d'estomac sur la sécrétion pancréatique C.R. Séanc Soc Biol 116:196-198 1934
- 15 Blair E L Brown J C Harper A A and Scratcherd T A gastric phase of pancreatic secretion *J Physiol* 184: 821-824 1966
- 16 Blondlot Traite analytique de la Digestion Paris 1843
- 17 Bloom H S Jacobson E D and Grossman M I Validation of dilution indicators in the stomach *Gastroenterology* 52: 205-210 1967
- 18 Bloom S R Mortimer C H Thorne M D Besser G M Hall H Gomez-Pan A Roy W M Russel R C G Coy D H Kestin A J and Schally A V Inhibition of gastrin and gastric acid secretion by growth-hormone release inhibiting hormone *Lancet*, II:1106-1109 1974
- 19 Bloom S R and Polak J H Gut hormone overview pp 3-18 in Bloom S R (ed) *GUT Hormones* Churchill Livingstone Edinburgh London and New York 1978
- 20 Brandeborg O Brandeborg H and Christensen N J The role of beta adrenergic receptor in the secretion of gastrin: studies in normal subjects and in patients with duodenal ulcers *Europ J Clin Invest* 6:395-401 1976
- 21 Brandeborg O Christensen N J Løvgreen H A Brandeborg H and Rehfald J Increased sensitivity of gastrin release to adrenaline in duodenal ulcer *Gut* 19 202-206 1978
- 22 Brown M and Vile W Effects of Neurotensin and substance P on plasma insulin glucagon and glucose levels *Endocrinology* 98:819-822 1976
- 23 Bulbring E and Cross A The release of 5-HT in relation to pressure exerted on the intestinal mucosa *J Physiol* 146 18-28 1959
- 24 Caldera R Ferreri C Romussi M Bortoli L Gandini S and Curterelli G Effect of dopamine infusion on gastric and pancreatic secretion and on gastrin release in man *Gut*, 19 724-728 1978

- 25 Chayvialle J A Miyata M Rayford Ph and Thompson J  
Effect of fundic distension on vasoactive intestinal peptide in dogs *Scand J Gastroent.* suppl 49 15:38 (The 2nd International Symposium on Gastrointestinal Hormones)
- 26 Cox A J Stomach size and its relation to chronic peptic ulcer *AMA Arch Path* 54:407-422 1952
- 27 Creutzfeldt W Arnold R Creutzfeldt C and Track N S  
Mucosal gastrin concentration molecular forms of gastrin number and ultrastructure of G-cells in patients with duodenal ulcer *Gut* 17:745-754 1976
- 28 Curwain B P and Holton P The effects of isoprenaline and noradrenaline on pentagastrin-stimulated gastric acid secretion and mucosal blood flow in the dog *Br J Pharmacol* 46:225-233 1972
- 29 Debas H T Konturek S J Walsh J H and Grossman M I  
Proof of a pylorooxyntic reflex for stimulation of acid secretion *Gastroenterology* 66:526-532 1974
- 30 Debas H T Walsh J H and Grossman M I Evidence for oxyntopyloric reflex for release of antral gastrin *Gastroenterology* 68:687-690 1975
- 31 Debas H T Walsh J H and Grossman M I Mechanisms for release of antral gastrin In *Gastrointestinal Hormones* ed J C Thompson Austin University of Texas Press p 425-435 1975
- 32 Debas H T and Yasaguchi T Evidence for pyloropancreatic reflex for pancreatic exocrine secretion *Am J Physiol* 234 E 468-471 1978
- 33 Dragstedt L B Peptic ulcer An abnormality in gastric secretion *Am J Surg* 117 143-156 1969
- 34 Edwards A V and Bloom S R Nervous control of pancreatic hormones In *GUT Hormones* ed S R Bloom Churchill and Livingstone Edinburgh London and New York pp 394-405 1978
- 35 Ekke S Borg I and Fyrd B Antral and duodenal gastrin activity in non-ulcer and ulcer patient *Scand J Gastroent.* 6 39-43 1971
- 36 Fahrenkrug J Haglund U Jodal M. Lundgren O Olbe L and Scheffalitzky de Muckadell O B Nervous release of vasoactive intestinal polypeptide in the gastrointestinal tract of cats possible physiologic implication *J Physiol (London)* 284 291-305 1978

- 37 Farooq O and Walsh J H Atropine enhances serum gastrin response to insulin in man *Gastroenterology* 68:662-666 1975
- 38 Faeth S Hultén L Lundgren O and Nordgren S Vascular responses to mechanical stimulation of the mucosa of the cat colon *Acta Physiol Scand* 101:98-104 1977
- 39 Feldman H Richardson C T Taylor I L and Walsh J H Effect of atropine on vagal release of gastrin and pancreatic polypeptide *J Clin Invest* 63:294-298 1979
- 40 Flemström E Effect of catecholamines  $Ca^{++}$  and gastrin on gastric  $HCO_3$  secretion *Proc Symp Gastric Ion Transport Uppsala 1977 Acta Physiol Scand special suppl* 31 90 1978
- 41 Floyd K and Harrison J F II Splanchnic mechanoreceptors in the dog *Quart. J. Exp. Physiol.* 59:361-366 1974
- 42 Fordtran J S and Walsh J H Gastric acid secretion rate and buffer content of the stomach after eating *J Clin Invest* 52:645-657 1973
- 43 Gershon H Drakontides A and Ross L Serotonin synthesis and release from the myenteric plexus of the mouse intestine *Science* 149:197-199 1965
- 44 Gillespie I E and Grossman H I Inhibitory effect of secretin and cholecystokinin on Heidenhain pouch responses to gastrin extract and histamin *Gut* 5:342-345 1964
- 45 Gottrup F and Ørnholt J Inhibition of gastric acid secretion in dogs by a new sympathomimetic drug *Scand J Gastroent* 13 337-343 1978
- 46 Gowers W II The automatic action of the sphincter ani *Proc Royal Soc. London* 11 77-84 1877
- 47 Greenlee H B Longh E H Guerrero F S Nelson A L El-Bedr A L and Dragstedt L Inhibitory effect of pancreatic secretin on gastric secretion *Amer J. Physiol* 190:396-402 1957
- 48 Gross M A Isenberg J I Hogan B and Samloff I Effect of f t on meal-stimulated duodenal acid load duodenal pepsin load and serum gastrin in duodenal ulcer and normal subjects *Gastroenterology* 75 357-362 1978

- 49 Grossman M I Stimulation of secretion of acid by distension of denervated fundic pouches in dogs *Gastroenterology* 41:385-390 1961
- 50 Grossman M I Secretion of acid and pepsin in response to distension of vagally innervated fundic gland area *Gastroenterology* 42:718-721 1962
- 51 Grützinger U Berggårdh S and Olbe L Effect of fundic distension on gastric acid secretion in man *Gut* 18:105-110 1977
- 52 Grützinger U Rehfeld J F and Olbe L Is there an oxyntopyloric reflex for release of gastrin in man? *Gastroenterology* 73:753-757 1977
- 53 Gustavsson S and Lundqvist M Participation of entral bombesin in the local regulation of gastrin release *Acta Endocrin* 88:339-346 1978
- 54 Harper A A Kidd C and Scratcherd T Vago-vagal reflex effects on gastric and pancreatic secretion and gastrointestinal motility *J Physiol* (London) 148:417-436 1959
- 55 Heidenheim R Ueber die Absonderung der Fundusdrüsen des Magens *Pflüger's Arch ges Physiol* 19:148 1879
- 56 Hillarp N A The construction and functional organization of the autonomic innervation apparatus *Acta Physiol. Scand* suppl 157 46 1-38 1959
- 57 Hirschowitz B I and Sachs G Lessons from the intact stomach In *Gastric Secretion* ed Georg Sachs Academic Press New York and London pp 7-33 1972
- 58 Hogben C A M Schenker L S Tocco D J and Brody E B Absorption of drugs from the stomach II The human *J Pharm. Exp. Ther* 120 540-545 1967
- 59 Hultén L Extrinsic nervous control of colonic motility and blood flow *Acta Physiol. Scand* suppl 335 1969
- 60 Iggo A Tension receptors in the stomach and the urinary bladder *J Physiol.* (London) 128:593-607 1955
- 61 Iggo A Gastro-intestinal tension receptors with unmyelinated afferent fibres in the vagus of the cat *Quart. J. Exp. Physiol* 42 130-143 1957
- 62 Iggo A Gastric mucosal chemoreceptors with vagal afferent fibres in the cat *Quart. J. Exp. Physiol* 42 398-409 1957

- 63 Isenberg J I Grossman H I Maxwell V and Walsh J H  
Increased sensitivity to stimulation of acid secretion by  
pentagastrin in duodenal ulcer *J Clin Invest* 55:330-  
337 1975
- 64 Ivey K J and Schedl H P Gastric nonabsorbable indicators  
for studies in man *Gastroenterology* 59:234-239 1970
- 65 Jacobson E D Priscolline stimulation of gastric secretion  
*Fed Proc* 20:251 1961
- 66 Jansson G Extrinsic nervous control of gastric motility  
An experimental study in the cat *Acta Physiol Scand*  
suppl 326 1969
- 67 Johnson L R and Grossman H I Effects of fat secretin and  
cholecystokinin on histamine stimulated gastric acid secre-  
tion *Amer J Physiol* 216:1176-1179 1969
- 68 Johnson L R and Grossman H I Characteristics of inhibi-  
tion of gastric secretion by secretin *Amer J Physiol*  
217:1401 1969
- 69 Johnson L R and Grossman H I Intestinal hormones as inhi-  
bitors of gastric acid secretion *Gastroenterology* 60 120-  
144 1971
- 70 Johnston D and Jepson K Use of pentagastrin in a test of  
gastric acid secretion *Lancet* 2:585-588 1967
- 71 Kihl B and Olbe L Inhibition of pentagastrin-stimulated  
gastric acid secretion by graded intraduodenal administra-  
tion of l l ic acid in man To be published
- 72 Klemensievi J Wiener Sitzungsab 1875 LXXI 249; Jahrb  
d Fort d Tierchem v 162 1875
- 73 Kock N G An experimental analysis of mechanisms engaged in  
reflex inhibition of intestinal motility *Acta Physiol*  
*Scand* suppl 164 1959
- 74 Konturek S J and Olewsky J The effect of cholinergic and  
adrenergic blockage on basal and pentagastrin-induced acid  
secretion *Scand J Gastroent* 4:13-16 1969
- 75 Kreulen D L and Szurazowski J H Reflex pathways in the  
abdominal prevertebral ganglia: Evidence for colo-colic  
inhibitory reflex *J Physiol* 295 21-32 1979

- 76 Larsson L I Sundler F and Håkansson E Pancreatic polypeptide - a postulated new hormone: Identification of its cellular storage site by light and electron microscopic immunocytochemistry. *Diabetologia* 12:211-226 1976
- 77 Lee C Y The effect of stimulation of extrinsic nerves on peristalsis and the release of 5-HT in the large intestine of the guinea pig and of the rabbit. *J Physiol* 152:405-418 1960
- 78 Leek M F Abdominal visceral receptors. Handbook of sensory physiology vol III part I: Enteroreceptors ed E Neil Springer Verlag Berlin pp 113-160 1971
- 79 Lillehei C W and Wangersteen H H Effect of celiac ganglionectomy upon experimental peptic ulcer formation. *Proc Soc Exp Biol Med* 68:369-372 1948
- 80 Lin R K S Ivy A C and McCarthy J E Contributions to the physiology of gastric secretion. *Quart. J Exp Physiol* 15:13-33 1925
- 81 Magee D F and Hu C Y Heidenhain pouch distension as a stimulus for acid and pepsin secretion. *Ann. Surg* 182:121-123 1975
- 82 Magee D F Gastric distension and the external secretion of the pancreas. *J Physiol* 149:76P-77P 1959
- 83 Meklouf G M and Said S I The effect of vasoactive intestinal polypeptide (VIP) on digestive and hormonal function. In *Thompson Gastrointestinal Hormones* pp 599-610 (University of Texas Press Austin 1975)
- 84 Meklouf G M Distinct mechanisms for stimulation of intestinal secretion by vasoactive intestinal peptide (VIP) and glucagon. *Gastroenterology* 72:368-369 1977
- 85 Melagelade J R Longstreth G F Deering T B Summerskill W H J and Vay Liang W Gastric secretion and emptying after ordinary meals in duodenal ulcer. *Gastroenterology* 73:989-994 1977
- 86 Hartnson J Studies on the efferent vagal control of the stomach. *Acta Physiol Scand* suppl 255 1963
- 87 Herzoff J H The effect of metoclopramide on human gastric emptying and secretion. *Acta Gastroenterol. Latinoam* 6:55-61 1974
- 88 Herzoff J H Hofraro J A Ferreira J A and Herzoff J C The effect of metoclopramide on human gastric secretion (abstr). *Gastroenterology* 66:743 1974

- 89 Moll H and Flint E R The depressive influence of the sympathetic nerves on gastric acidity *Bell J Surg* 16: 283-307 1928/29
- 90 Moraes H F Nyhus L H Kalahaine N G Bambeck C Th and Das Gupta T K Role of the sympathetic nervous system in peptic ulcer production in rats *Surgery* 83:194-199 1978
- 91 Morrison J F B Mechanoreceptors in the region of the mesentery with A-delta and C fibres in the splanchnic nerves of the cat *J Physiol (London)* 226:100P-101P 1972
- 92 Oberhelman H A Woodward E R Smith C A and Dragstedt L Effect of sympathectomy on gastric secretion in total pouch dogs *Amer J Physiol* 166:679-685 1951
- 93 Paintal A S A study of gastric stretch receptors Their role in the peripheral mechanism of satiation of hunger and thirst *J Physiol (London)* 126:255-270 1934
- 94 Pavlov I P The work of Digestive Glands 2nd ed London 1910
- 95 Penner A Hollander F and Selzsann H The gastric absorption of phenol red in humans *Amer J Dig Dis* 5:657-661 1938
- 96 Petersen H and Myren J Pentagastrin dose-response in peptic ulcer diseases *Scand J Gastroent.* 10:705-714 1975
- 97 Phillip J Domachke S Domachke W Urbach H J Reiss M and Deuling J Inhibition by somatostatin of gastrin release and gastric acid response to meals and to pentagastrin in man *Scand J Gastroent.* 12:261-265 1977
- 98 Porte D Jr Gurel L Seydoux J Kanazawa Y and Posternak J Neural regulation of insulin secretion in the dog *J Clin Invest.* 52:210-214 1973
- 99 Prosser M M Effect of vagotomy on the acid response to gastric distension *Canad J Physiol Pharmacol* 48:670-674 1976
- 100 Raptin E Dollinger H C von Berger L Schlegel W Schreiber K E and Pfeiffer E F Effects of somatostatin on gastric secretion and gastrin release in man *Digestion* 13:15-26 1975
- 101 Réaumur H Acad roy d sci de Paris 1752
- 102 Richardson C T Walsh J H Hicks H I and Fordtran J S Studies on acid secretion in normal human subjects *J Clin Invest.* 58 623-631 1976

- 103 Roland M. Berstad A and Llivåg I Effect of carbacholine and urocholine on pentagastrin-stimulated gastric acid secretion in healthy subjects *Scand J Gastroent.* 10:357-362 1975
- 104 Rottler J I and Rimoin D L Peptic ulcer disease - a heterogeneous group of disorders? *Gastroenterology* 73:604-607 1977
- 105 Schrupf E and Stadess J Effect of gastric distension on motility and plasma gastrin concentration before and after secretin administration *Scand J Gastroent.* 9:119-122 1974
- 106 Schüle I In wie weit stimmen die Experimente von Pavlov an Hunde mit den Befunden an normalen menschlichen Magen überein? II Ueber die Beeinflussung der Salzsäurecurve durch die Qualität der Nahrung? *Deutsches Archiv für klinische Medizin* 71:111-132 1901
- 107 Schwartz T W Grützinger U Schön I -M and Olbe L Vago-vagal stimulation of pancreatic polypeptide secretion by graded distension of the gastric fundus and the antrum in man *Digestion* 19:307-314 1979
- 108 Schwartz T W Rehfeld J F Stadil F Larsson L I Chance R E and Moon N Pancreatic polypeptide responses to food in duodenal ulcer patients before and after vagotomy *Lancet*, I:1102-1103 1976
- 109 Schwartz T W Stenquist B Olbe L and Stadil F Synchronous oscillations in the basal secretion of pancreatic-polypeptide and gastric acid. Depressions by cholinergic blockade of pancreatic-polypeptide concentrations in plasma *Gastroenterology* 76:14-19 1979
- 110 Soares E C Zetrika S. and Walsh J Acid secretion and serum gastrin in relation to graded intragastric pressures in man *Gastroenterology* 72:676-679 1979
- 111 Spillanzani Expériences sur la digestion de l'homme et de différentes espèces d'animaux Genève 1783
- 112 Stenquist B Knutson U Rönberg O and Olbe L The effect of intragastric pH-variations on the gastric acid response to insulin hypoglycaemia in healthy subjects and duodenal ulcer patients *Scand J Gastroent.* 12:169-175 1977



- 113 Stenquist H Rehfeld J F and Olbe L Effect of proximal gastric vagotomy and anticholinergics on the acid and gastrin responses to sham feeding in duodenal ulcer patients *Gut* 20:1020-1027 1979
- 114 Taylor I L Impicatore M Carter D C and Walsh J H Effect of atropine and vagotomy on the pancreatic polypeptide response to meal in dogs *Am J Physiol* 235:E 443-447 1978
- 115 Taylor I L Walsh J H Carter D Wood J and Grossman M I Effects of atropine and betanecol on bombesin-stimulated release of pancreatic polypeptide and gastrin in dog *Gastroenterology* 77:714-718 1979
- 116 Taylor I V Elder J B Ganguli P C and Gillespie I E Comparison of an intragastric method of estimating acid output with the pentagastrin test in normal and duodenal ulcer subjects *Gut* 19:865-869 1978
- 117 Thompson J C and Swierczek J S Acid and endocrine responses to meals varying in pH in normal and duodenal ulcer subjects *Ann Surg* 186:541-548 1977
- 118 Thompson J C Hormonal influences on gastric secretion In *Advances in Surgery* Year Book Medical Publishers 12:53-83 1978
- 119 Thorpe C D Frusca R A Bass P and Hug C C Jr Effects of catecholamines on acid secretion by bullfrog isolated gastric mucosa *Surg Forum* 22:317 1971
- 120 Tintobello A Caldera R Ferrari C Rossetti M and Berti L Effects of dopamine and of the dopaminergic drug bromocriptine on gastric acid and gastric secretion in man *Abstr VI World Congress of Gastroenterology Madrid* p 20 June 1978
- 121 Tiscornia H M The neural control of exocrine and endocrine pancreas *Amer J Gastroent* 67:541-560 1977
- 122 Uvnäs-Wallensten K Effendi S and Luft R Vagal release of somatostatin into the entral lumen of cats *Acta Physiol Scand* 99:126-128 1977
- 123 Valenzuel J E and Grossman M I Dopamine stimulates pancreatic secretion and inhibits gastric secretion in dogs (abstr) *Clin. Res* 15:292A 1976

- 124 Valenzuela J E Defilippi C Diaz D Mari E and Merino Y Effect of dopamine on human gastric and pancreatic secretion *Gastroenterology* 76:323-326 1979
- 125 Vizi E M Interaction between adrenergic and cholinergic systems: presynaptic inhibitory effect of Noradrenaline on Acetylcholine release *Journal of Neural Transmission* suppl XI pp 61-78 ed B Csillik and J Ariens Kapper by Springer Verlag 1974
- 126 Walsh J H and Grossman M I Gastrin *New Eng J Med* 292:134-138A and 1377-1384 1975
- 127 Walsh J H Richardson C T and Fordtran J S pH dependence of acid secretion and gastrin release in normal and ulcer subjects *J Clin. Invest* 55:462-468 1975
- 128 Walsh J H Yalow R S and Berson S A The effect of atropine on plasma gastrin response to feeding *Gastroenterology* 60:16-21 1971
- 129 White T T McAlejandro R A and Magee D F The effect of gastric distension on duodenal aspirates in man *Gastroenterology* 44:48-51 1963
- 130 White T T Hayama T and Magee D F Alternate nervous pathways for the gastropancreatic reflex *Gastroenterology* 39:615-618 1960
- 131 White T T Lundh G and Magee D F Evidence for existence of gastropancreatic reflex *Amer J Physiol* 198:725-728 1960
- 132 White T T and Magee D F The splanchnic nerves and the gastropancreatic reflex *Gastroenterology* 45:698 1963
- 133 Wikberg J E S The pharmacological classification of adrenergic  $\alpha_1$  and  $\alpha_2$  receptors and their mechanisms of action *Acta Physiol. Scand* suppl 468 1979
- 134 Wormsley E G and Grossman M I Maximal histalog test in control subject and patients with peptic ulcer *Gut*, 6 427-435 1965
- 135 Yamaguchi T and Debas H T Antral distension with acid inhibits gastric acid secretion (abstr.) *Gastroenterology* 72:1152 1977
- 136 Zilinsky Sav tech Rannion biol & St Petersburg 1914 LXXVII 50 Russian Journ Physiol 1922 1 (quoted from Physiol Abstr 1922 VII 3292)







FACTA

RELATION BETWEEN

BENEFITUELUS

LUND 1880



**ACTA PHYSIOLOGICA SCANDINAVICA**

**Supplementum 483**

**From the Institute of Physiology and Biophysics**

**University of Lund**

**Sweden**

**RELATION BETWEEN**

**MECHANICAL AND MORPHOLOGICAL CHARACTERISTICS**

**IN URINARY BLADDER SMOOTH MUSCLE**

**BY**

**BENGT UVELIUS**

**LUND 1980**



This summary contains apart from previously unpublished data results from the following papers

- I UVELIUS B 1976 Isometric and isotonic length-tension relations and variations in cell length in longitudinal smooth muscle from rabbit urinary bladder. *Acta Physiol Scand* 97: 1-12
- II UVELIUS B 1977 Influence of muscle length on the force-velocity relation of K<sup>+</sup>-contractures in smooth muscle from rabbit urinary bladder. *Acta Physiol Scand* 101: 270-277
- III JOHANSSON B, HELLSTRAND P & UVELIUS B 1978 Responses of smooth muscle to quick load change studied at high time resolution. *Blood Vessels* 15: 65-82
- IV UVELIUS B 1979 Shortening velocity, active force and homogeneity of contraction during electrically evoked twitches in smooth muscle from rabbit urinary bladder. *Acta Physiol Scand* 106: 481-486
- V UVELIUS B & Hellstrand P 1980 Effects of phasic and tonic activation on contraction dynamics in smooth muscle. *Acta Physiol Scand* in press
- VI UVELIUS B & GABELLA G 1980 Relation between cell length and force production in urinary bladder smooth muscle. Submitted to *Acta Physiol Scand*

The papers are referred to in the text by their Roman numerals

## CONTENTS

1	GENERAL INTRODUCTION	5
2	MORPHOLOGICAL ASPECTS	8
2:1	Introduction	8
2:2	Outline of methods	8
2:3	Variations in the muscle layer thickness with changes in bladder volume	10
2:4	Rearrangement of muscle bundles with increasing bladder volume	11
2:5	Muscle cell length in relation to bladder distension	12
2:6	Number of cells/unit area. Dependence on bladder volume	18
2:7	Effects of passive stretch on cell volume	19
2:8	General morphological description of isolated strips used for mechanical experiments	0
3	MECHANICAL PROPERTIES	21
3:1	Introduction	22
3:2	Outline of methods	24
3:3	Length-tension relations of bladder strips in vitro	25
3:4	Calculation of cellular length-active tension relations	29
3:5	Isotonic behaviour after quick release	33
3:6	Influence of muscle length on force-velocity relations	35
3:7	Effects of different modes of activation on contraction dynamics	36
3:8	Single twitches. Shortening velocity and active force	38
4	EFFECTS OF PROLONGED BLADDER DISTENSION ON LENGTH-TENSION RELATIONS	40
5	GENERAL DISCUSSION	43
6	SUMMARY	46
7	REFERENCES	47



## 1 GENERAL INTRODUCTION

Due to intense research over many years our present knowledge is large regarding morphology energetics mechanical properties etc of skeletal muscle. The sliding filament concept of contraction was established some 25 years ago (for review see e.g. Huxley 1971). Research on smooth muscle mechanics has been hampered for several reasons. The experimental procedures have been difficult to standardize and it has been difficult to obtain reproducible results. Katz showed however already in 1939 that Hill's (1939) equation is applicable also to smooth muscle by experiments on tortoise retractor penis muscle. In more recent years Katz' results have been confirmed and extended for a variety of mammalian smooth muscles (e.g. Åberg & Axelsson 1965, Hardung & Laszt 1966, Stephens, Kroeger & Mahta 1969, Mashima & Handa 1969, Gordon & Siegelman 1971, Hellstrand & Johansson 1975). From these studies it can be seen that smooth muscle in general has lower maximal shortening velocity ( $V_{max}$ ) than skeletal muscle and that isometric tension for most preparations is of the same magnitude as for skeletal muscle. There is great variation between results obtained from different smooth muscles regarding  $V_{max}$ . Whereas  $V_{max}$  was only 0.03 lengths/s for rabbit taenia coli at 22°C (Mashima & Handa 1969) it was 0.74 l/s for rat portal vein at 37°C (Hellstrand & Johansson 1975). Part of this difference is probably due to different methods (afterloaded isotonic contractions or quick release) and temperature but most probably different smooth muscles possess individual characteristics.

The mechanical behaviour of a smooth muscle depends (among other things) on the characteristics of the contractile proteins and on the relation between neighbouring cells since smooth muscle preparations are multicellular. During recent years it has been shown (see Somlyo & Somlyo 1975) that smooth muscle contains thick and thin myofilaments although not as regularly arranged as in skeletal muscle. The intercellular relations are complicated. In taenia coli from guinea pig (Gabella 1976c) the cells are anchored to each other and to collagen fibrils along the cell surface and at the end of the cells complicated interdigitations are observed.

If mechanical properties determined for smooth muscle preparations are to be considered relevant for the individual cell one has to know how the cells behave in comparison to the whole preparation e.g. how the lengths of the cells change in proportion to a length perturbation of the whole

preparation. The most straight-forward way to avoid this difficulty is to study the behaviour of isolated individual cells. It is possible to obtain viable single cells from toad stomach digested with trypsin and collagenase (see review by Fay Cooke & Canada 1976). Such cells can be mounted for recording of contractile force during activation for instance by electrical stimulation. It is not certain however that the mechanical output of an isolated cell represents the behaviour of a cell in situ anchored as it is not only at its ends but also laterally to neighbouring cells and to collagen fibrils present in the space between the cells.

Another way to obtain information regarding the mechanics of the individual cells is to perform the relevant experiments on multicellular preparations in which the behaviour of the individual cells in relation to the preparation as a whole is known. With this approach and the above mentioned background the present study was performed.

#### Scope of present investigation

The preparations used are rabbit and guinea pig urinary bladder. Some experiments are performed on whole bladders in vivo and others on strips of longitudinal bladder muscle in vitro. The reason for the choice of this preparation (which has not been used much previously for mechanical studies) was mainly that the length of the isolated preparations could be increased considerably without any marked increase in passive tension. The bladder as a densely innervated organ with only minor spontaneous activity is also easy to control and stimulate. Some comparison is made with results on rat portal vein which has considerable passive tension and a phasic spontaneous activity. This is done with the notion that conclusions regarding smooth muscle in general can be drawn with greater certainty if comparative results are obtained from different smooth muscle types.

The results of the morphological and morphometrical studies of the bladder preparations are accounted for in chapter 2. The effects of bladder distension on cell length and cell diameter in whole bladders and of length changes in isolated strips are shown.

Chapter 3 deals with the mechanics of bladder muscle. Length-tension relations for single smooth muscle cells are calculated from volume-actin-pressure relations in vivo. An analysis is given of the applicability of the isometric quick release method (Jewell & Wilkie 1960) for the study of

mechanics of isolated strips. The behaviour of the muscle strips during active shortening and the effect on this of different modes of stimulation are investigated. A study of the capability of the bladders to adapt to an introduced residual volume is given in chapter 4. Chapter 5 contains a general discussion and a summary.

## 2 MORPHOLOGICAL ASPECTS

### 2.1 Introduction

The mammalian smooth muscle cell in general is thin and slender. The dimensions vary depending on the organ and on the functional state (see rev by Burnstock 1970). The mid-cell diameter ranges from 1.5 to 8  $\mu\text{m}$  and the length from 30 to 450  $\mu\text{m}$ . Earlier ultrastructural studies of urinary bladder smooth muscle cells (Nagesawa & Suzuki 1967, Larsen 1977) show that bladder cells conform to this general description. The length and diameter of rabbit bladder cells were estimated to be about 200  $\mu\text{m}$  and 5  $\mu\text{m}$  for relaxed and 100  $\mu\text{m}$  and 9  $\mu\text{m}$  for contracted cells (Larsen 1977). Both thick and thin myofilaments were observed. Available data is however not sufficient for a proper understanding of cellular events in relation to the behaviour of the bladder as a whole. The aim of the studies reviewed in this chapter is to provide such quantitative information that will be necessary for the interpretation of the results from the mechanical experiments in chapter 3. From a description of the remodelling of the muscle wall when the bladder is inflated (2.3-2.4) attention will be focused on the behaviour of the single muscle cell in situ. Cell length (2.5) and number of cells per  $\text{mm}^2$  (2.6) will be described in relation to bladder radius. In 2.7 an attempt will be made to determine how cell volume is affected by bladder distension. Finally (2.8) morphological characteristics are given for the isolated bladder strips used in chapter 3.

### 2.2 Outline of methods

Adult guinea-pigs (body weight 300-500 g) and rabbits (body weight 2500-3500 g) were used. The general processing is outlined below. There will be divergences from this scheme in section 2.8 but they will be accounted for there.

The animal was killed and the bladder was excised, emptied and transferred to oxygenated Krebs solution (paper VI). The urethra was then cannulated and the bladder slowly filled to a desired volume either through a gravity fall or by stepwise injections with a syringe connected to the cannula. Filling could take up to 30 min for the highest volumes. The bladder was then transferred to glutaraldehyde solution at room temperature. After about 10 min the bladder was cut open, divided into identifiable parts which were transferred to fresh fixative. Some bladders were fixed with their content of urine immediately after ligation of the urethra and removal

from the animal. After 10 min of fixative the bladder was slit open and the content volume was measured. The bladders were then treated as described in the foregoing. The preservation of fine structures was not good in these bladders perhaps due to leakage of urine into the bladder wall during fixation.

After the fixation period (2-6 h) the specimens were immersed for 1-2 h in 2 % osmium tetroxide and then transferred for 30 min to a 1 % aqueous solution of uranyl acetate. After dehydration with ethanol and propoxypropane the specimens were infiltrated with Araldite. After polymerization suitable blocks were cut with glass knives. Sections 1.5 - 3.5  $\mu$ m thick for light microscopy were collected on microscope slides, examined and photographed unstained in a phase contrast microscope. Sections for electron microscopy were collected on grids, stained with lead citrate and examined in a Philips 300 electron microscope. All histological observations were carried out on detrusor muscle from the mid-ventral part of the bladder.

When cross sections should be made on muscle bundles in a block, the exact direction of the bundle was visualized under a dissection microscope and appropriate cuts could be made. Bladders with small content volumes were not translucent enough to make it possible to see the bundles. Under such circumstances it was necessary to make serial sections for phase contrast microscopy to ensure that a bundle was at right angle to the direction of the cuts. Generally a bundle was followed over at least 1  $\mu$ m.

Bladder radius will be expressed as bladder outer radius. This is calculated from the bladder outer volume which is obtained by the addition of the bladder wall volume to the content volume. The reason for doing this is that the muscle layer is much closer to the outer than to the inner surface due to a submucosa of considerable thickness. In other experiments the weights of guinea pig bladders were found to be about 0.3 g and therefore 0.3 ml is added to all content volumes in order to obtain outer volumes. Mean rabbit bladder weight was 1.8 g and 1.8 ml is added to the content volumes in rabbit bladders in order to obtain outer volumes.

Bladder radius and circumference are calculated from bladder volume assuming spherical form. The guinea-pig bladder is in fact almost spherical at all volumes studied (0 - 6.7 ml). Thus the rabbit bladder is slightly elongated.

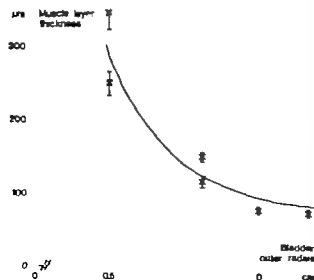


Quantitative results are given as mean  $\pm$  standard error (SE). Number of observations are given in parenthesis.

**3 Variation in the muscle layer thickness with changes in bladder volume**

If distension of the bladder portion that is used in this study (the midventral portion) is to be calculated from the bladder volume, the assumption has to be made that all parts of the bladder wall are stretched by the same relative amount in response to increases in bladder volume. It can be expected a priori that one part of the bladder wall, namely the trigonum, is more resistant to stretch than the rest, but the trigonum constitutes only a small portion of the bladder wall. In this section the applicability of the assumption that all parts of the guinea pig bladder are stretched to the same extent will be considered.

Araldite-embedded midventral portions from 6 guinea pig bladders were used. The bladders were fixed with content volumes between 0.25 ml and 6.7 ml. The muscle wall thickness was measured with a calibrated eye piece in the phase-contrast microscope. 15 determinations were performed for each of the six bladders. The results are shown in Fig. 1. It is seen that the muscle wall thickness decreases when the bladder outer radius is increased. At a bladder outer radius of 0.51 cm (corresponding to a content volume of 0.25 ml and an outer volume of 0.55 ml)



**Fig. 1** Thickness of total muscle layer of 6 guinea-pig bladders filled to different volumes. Bladder outer radius represents radius of the content assuming spherical shape plus total bladder wall thickness. Full line indicates expected behaviour calculated from the volume of the muscle layer at the lowest bladder volume assuming that all parts of the bladder are equally distensible. Each point represents mean of 15 thickness determinations.

the mean muscle layer thickness of the 2 bladders is 300  $\mu\text{m}$ . This gives a calculated total volume of the muscle layer of 92  $\text{mm}^3$ . If all parts of the bladder wall are stretched to the same extent when the bladder is inflated and if the volume of the muscle layer can be considered independent of content volume, muscle layer thickness should vary in accordance with the full line in Fig. 1. The thickness measurements agree well with what is to be expected if the above mentioned assumptions are relevant.

The results in this section thus show that a certain relative increase in guinea pig bladder radius results in the same relative length change in the particular segment of the bladder wall used in the experiments (the mid-ventral portion).

#### 2.4. Rearrangement of muscle bundles with increasing bladder volume

The muscle wall of the bladder is considered to consist of three ill-defined layers: an outer and an inner longitudinal and an intermediate circular layer (see e.g. Ham 1969). The circular layer is usually the thickest. This description seems to be valid also for the bladders used in this study. Each layer seems to consist of muscle bundles separated from each other by connective tissue. The direction of the bundles in each layer is not uniform. Bundles with a direction almost at right angle to the others are often found in the different layers. The expressions circular and longitudinal layer therefore express only the general direction of the muscle bundles within each layer.

The thickness of the muscle layer as a whole decreases when the bladder is inflated as shown in section 2.3. In the phase-contrast microscope it can be seen that cross-sectioned bundles in sections from distended bladders seem to be somewhat flattened when compared to bundles from bladders with a low content volume. This flattening is however not prominent. The profiles of individual cells in cross-sectioned bundles are, by judging from the electron micrographs, somewhat flattened in the distended bladders but much less so than would be expected if the bundles were stretched laterally in proportion to the increase in bladder diameter.

The phase-contrast micrographs (VI) suggest that the muscle bundles spread laterally when the bladder is inflated, i.e. that the radial number of bundles decreases with increasing bladder volume. This is a general

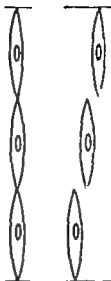
impression for the 6 guinea-pig and 8 rabbit bladders studied. If this is correct the number of muscle cells across the thickness of the wall would decrease when the bladder is inflated. Therefore the number of muscle cells across the full thickness of the muscle coat was determined by counting how many muscle cells were intersected by radial lines drawn orthogonally through the wall. These determinations were performed either on phase-contrast micrographs or directly in the microscope. The results show that the radial number of cells decreases with increasing bladder radius for all volumes studied (VI). This is to be expected if, as suggested above, the muscle bundles spread laterally when the bladder is inflated.

There are two additional conclusions to be drawn from the results presented above. 1. It is difficult if not impossible to dissect out strips for in vitro experiments where all cells have the same direction. Even in strips from one muscle layer bundles with a considerable oblique direction in relation to the long axis of the preparation will be included. This will be of relevance for the in vitro determination of force per unit cross sectional area (see chapter 3). 2. As the muscle bundles in the wall tend to spread tangentially more than being flattened when the bladder is inflated, length variations for isolated strips (here the bundles are not flattened) will be a good model of the individual bundle in situ. If the bundles had become considerably flattened in situ the geometrical arrangements of the cells within a bundle might have changed with bladder volume. Then it would have been much more difficult to transfer results from strips in vitro to the whole bladder.

## 2.5 Muscle cell length in relation to bladder distension

If results from mechanical experiments on whole bladders or bladder strips are to be carried over to individual cells in the preparation, it is essential to know how the cells are coupled to each other. Fig. 2 shows schematically 2 extreme arrangements: 1. a series (SC) and parallel coupling (PC). Intermediates between these are also possible. If the individual cells in the figure produce the same force, then the force output of the model with 3 parallel coupled cells would be 3 times greater, whereas the SC preparation gives just the same force as one cell. If the length of the preparations is doubled by passive stretch, then the cell length in the SC model is also doubled, whereas cell length for the PC model increases to a much greater extent. These are drastic examples showing the necessity

Fig. 2 Alternative arrangements of muscle cells within a tissue. The cells coupled in parallel (right) produce per unit cross sectional area 3 times the force attained by the preparation with the cells coupled in series (left). The non-cellular force-transmitting structures in the right panel are assumed to be rigid. Modified from Murphy (1976)



of knowing the cellular coupling if cellular behaviour in intact preparations is to be calculated

If cell lengths are determined for preparations subjected to different degrees of stretch the cell length coupling can be determined (see Murphy, Briskin & Cohen 1977). Should the cells be coupled in series a direct proportionality between changes in preparation length and cell length is to be expected. Should they be parallel coupled the relative length change of the cells is greater than for the preparation as a whole, the magnitude being dependent on the degree of parallel coupling.

In order to study the dimensions of individual cells Anopolsky (1928) obtained suspensions of isolated smooth muscle cells from pig fallopian tube by maceration of unfixed tissue pieces in 30 % nitric acid. Cooke & Fay (1972) macerated glutaraldehyde fixed strips of guinea-pig taenia coli in 25 % KOH. The method used in paper 1 is a modification of the method devised by Anopolsky. Rabbit bladders were filled to desired content volumes and placed for 1 h in Ca-free Krebs solution containing 1.0 mM EGTA in order to chelate  $\text{Ca}^{2+}$  and thereby ensure that the cells are in a relaxed state. The bladders were filled in an isotonic buffered formaldehyde fixative. After 24 h the fixative longitudinal strips of the bladders

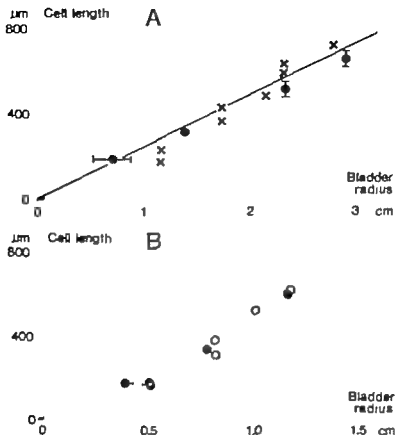
were dissected out. These strips were then placed in increasing concentrations of nitric acid 1/2 h in each starting with 0.6 % (roughly isosmolar). The strips were finally placed in 30 %  $\text{HNO}_3$  for 4 days. The rationale for the stepwise increase in concentration was to avoid possible effects on the cells such as shrinkage induced by drastic changes in osmolality of the bathing medium. When the macerated strips were gently stirred after 4 days in nitric acid a suspension of isolated cells and cell conglomerates was obtained. A drop of this suspension was placed on a slide and a cover slide was put on. Isolated individual cells were photographed and the lengths were measured on the prints. Only isolated intact symmetrically formed cells with well tapered ends were considered.

In order to ascertain that the muscle cells were not activated during fixation and that they did not shrink when they were placed in the nitric acid solutions tension of some longitudinal strips was measured continuously during these procedures. No tension developed which strongly suggests that the cells were fixed in a relaxed state.

Filled circles in Fig. 3A show the results of the cell length determinations. It is clearly seen that an increase in bladder radius is accompanied by a closely similar relative length change of the individual cells. The results agree qualitatively with those of Cooke & Fay (1972) on guinea-pig taenia coli although they have studied cells from only two preparation lengths. The results are also well in accordance with those on pig carotid artery reported by Murphy, Driska & Cohen (1977), Driska & Murphy (1978) and Driska, Denson & Murphy (1978).

There is some uncertainty regarding the reliability of the above study since the length of the cells might possibly change during the maceration of the strips despite that no tension development was observed. Such tension measurements can probably not account for all possible changes in cell length during the maceration especially not during the later phase when the cells begin to loosen from each other. It was also felt that length changes if they occurred might be of different importance at different degrees of stretch.

The most reliable data regarding cell length are most probably obtained from serial electron microscope sampling. Such studies have been done (e.g. Bennet & Rogers 1967, Merrillees 1968) but the approach has not been



**Fig. 3** Muscle cell length vs bladder outer radius. **A** Rabbit. Filled circles indicate determinations on nitric acid macerated bladders. 3 bladders at each volume. Vertical bars indicate  $\pm$  SE ( $n = 53-79$ ). Horizontal bars wall thickness. Crosses show morphometrically determined lengths of longitudinal muscle cells. The line shows the expected behaviour if the cells were coupled in series. **B** Guinea-pig. Open circles longitudinal muscle; filled circles circular muscle. For longitudinal muscle radius is given as outer radius; for circular muscle as inner radius. Points connected by broken lines indicate results from same bladder. From (I) and (VI).

applied to the study of different degrees of stretch. It would probably be too laborious to obtain a reasonable number of length determinations in such an investigation.

Halpern, Mulvany & Marshaw (1978) have used Nomarski interference contrast optics to measure distance between intracellular structures of the same muscle cell in segments of small intramural arteries in vitro. Increases

in internal circumference by up to 50 % in inactive vessels resulted in a nearly proportional increase in spacing between the intracellular markers. The results regarding the part of the muscle cell between the markers thus correspond well with those described above. These authors have however no results regarding the behaviour of the tapering ends of the cells.

Gabella (1976a) has devised a morphometric method to determine muscle cell length

$$\text{mean cell length} = \frac{\text{mean nucleus length} \times 100}{\% \text{ of cross-sectioned cell profiles containing nuclei}}$$

The advantage of this method is that the cells are left in situ thus minimizing the risk that length changes could occur. This method was applied to 6 guinea-pig and 8 rabbit bladders. Cross sections for EM were cut from identified longitudinal muscle bundles and the number of cross sectioned cells were counted as were the number of cells containing nuclear profiles. Then the tissue blocks were re-embedded in Araldite and tangential sections for phase-contrast microscopy were cut from the bundles chosen above. Lengths of nuclei with identifiable nuclear poles were measured with a calibrated eye piece. With guinea pig bladder the same method was used also for determining cell length in the circular muscle layer. The number of cross sectioned cell profiles counted in each determination ranged between 448 and 2252 and the number of nuclei between 26 and 115. The majority of the cell profiles are smooth indicating that these cells are fixed in a relaxed state. A fraction of the cells have however rough surfaces and protrusions which indicates that these fibres were fixed in an activated state (regarding such protrusions see e.g. Gabella 1976b).

The calculated cell length determinations are shown for rabbit bladder cells in Fig. 3A and they are similar to those obtained by nitric acid necration. It thus appears that the less tedious nitric acid method gives data as reliable as those obtained by the method of Gabella. The full line in Fig. 3A shows the expected cell length variations in relation to rabbit bladder radius if the cells were coupled in series. The experimental data fit closely to this line. According to Fig. 3B a certain relative increase in guinea pig bladder radius is accompanied by a slightly larger relative length increase of the muscle cells. This suggests that there may be some degree of parallel coupling in this preparation. The deviation from the

series coupling behaviour is however so small that the cells in the guinea pig bladder can be considered in general to be coupled in series

It is not known if smooth muscle cells shorten when activated if the preparation as a whole is kept at constant length. The bladders used for nitric acid maceration were pretreated with EGTA and no comparison was made with bladders fixed while activated. The morphometric determinations described above were done on bladders that were not depleted with  $\text{Ca}^{2+}$ ; the fixative itself had 0.5 mM  $\text{Ca}^{2+}$  added. The electron-micrographs also showed that some cells were fixed in an activated condition. Despite this cell length was almost the same as for the macerated inactive bladders. The cell length determinations by Driske & Murphy (1978) on pig carotid artery were done on K<sup>+</sup> activated strips alone. Mulvany and Marshaw (1979) were unable to measure distance between intracellular markers for activated mesenteric arteries in vitro due to movements within the preparation. They found however that nucleus length as on average little changed. This suggested according to these authors that cell length was on average not substantially affected by activation. Fig. 4 shows nucleus length vs bladder radius for rabbit bladder. It is seen that bladder radius and thus cell length can vary considerably without much change in length of the nucleus. At least for this preparation nucleus length does not seem to correlate well with cell length.

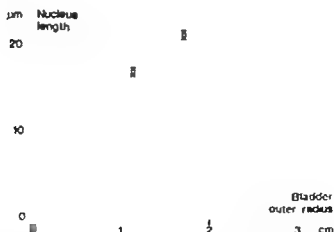


Fig. 4. Length of nuclei in longitudinal muscle from rabbit bladder. Each point represents mean value from 35 nuclei of one bladder. 8 bladders were used. Only nuclei with all identified clear zones around the nucleus were considered.



While no conclusive results exist regarding lengths of activated cells vs passive it my impression that such length changes if they occur are small. This statement based on the fact that no significant length difference was found between passive macerated bladder muscle cells and morphometrically determined cell lengths of bladders fixed in the presence of  $\text{Ca}^{2+}$  where some cells showed signs of being fixed while active (Fig. 3A).

#### 2:6 Number of cells/unit area Dependence on bladder volume

It was shown in section 2:5 that cell length increased in a bladder muscle preparation when the length of the preparation is increased. The number of cells per unit area (cell packing density) in cross-sectioned muscle bundles would then be expected to rise with increasing stretch of the preparation.

Results regarding cell packing density of guinea pig taenia coli strips fixed at different lengths have been reported (Gabella 1976b). In one experiment a taenia strip was fixed at resting length whereas a contiguous strip was allowed to shorten actively down to 24 % of resting length. Packing densities were 94000 cells  $\text{mm}^{-2}$  and 18000 cells  $\text{mm}^{-2}$  respectively. In a similar experiment the packing density was 105000  $\text{mm}^{-2}$  in the stretched strip and 37000  $\text{mm}^{-2}$  in the strip fixed when shortened to 40 % of initial length.

In the present study on guinea pig urinary bladder cross-sectioned muscle bundles were marked out on EM-plates (VI). The cell numbers were counted and the contours of the bundles were copied onto transparent film sheets. These contours were then cut out and weighed. By knowing the weight of a calibration area the cross sectional area of the bundles could be calculated. Fig. 5 gives the results. Each point is based on 184-405 cells. For the whole radius interval studied cell packing density increases when the bladder is inflated. The straight line obtained by linear regression analysis fits well ( $r = 0.91$ ) with the experimental data.

Only four rabbit bladders were studied. In the two preparations fixed with a content volume of 5 ml packing density in the longitudinal muscle was 86000 and 64000 per  $\text{mm}^2$  respectively. From the mean values of these (75000  $\text{mm}^2$ ) the figure at 20 ml would be expected to be 119000  $\text{mm}^2$  ( $4^{1/3} \cdot 75000$ ). The actual data for two bladders fixed at this volume were 117000 and 141000 (mean 129000) which fit close to the expected value.

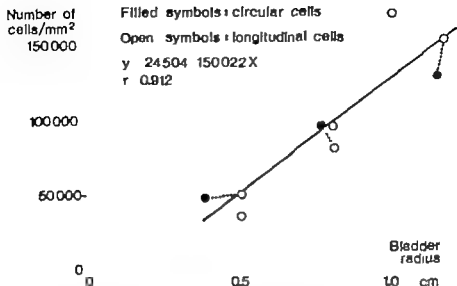


Fig 5 Number of cells per mm<sup>2</sup> cross-sectional area vs bladder outer radius. Broken lines connect values from same bladder. The best fitting line according to linear regression analysis is given. From VI.

The results in this section as well as those in section 2.5 (increase in cell length with bladder radius) suggest that no slippage between cells occur even at great distension of the bladder wall. The importance of this finding will be discussed in chapter 3 with regard to possible explanations for the broad and flat length-active tension curve that will be shown to exist for this type of muscle.

## 2.7 Effects of passive stretch on cell volume

According to Fay & Delise (1973) smooth muscle cells from toad stomach decrease in volume when they shorten actively. As the cells shortened to 30 % of their resting length the calculated cell volume decreased to 80 % of the initial value.

The results from paper VI reported under 2.5 and 3.6 permit us to speculate on the effect of bladder distension on cell volume. Fig 6 is a composite of the data in Figs 3B and 5. The relation between cell length and cell packing density is well approximated by a straight line in that a doubling of cell length is accompanied by a doubling of the cell packing density. This would indicate that cell volume does not change with length if the

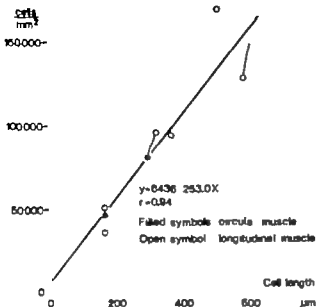


Fig 6 Number of cells per cross-sectional area vs cell length. The figure is composed of results from Fig 38 and Fig 39. Broken lines connect results from same bladder. Best fitting line obtained by linear regression analysis is shown.

amount of intercellular space was constant. The problem of the constancy of this space with changes in muscle length has not yet been investigated by use of specific tracers such as Co EDTA. However, microscope observations by Gabella (personal communication 1980) indicated that the size of the extracellular space is length-independent in guinea-pig taenia coli.

While the results of my own studies may not be conclusive on this point, they do suggest that cell volume does not change much when cell length is varied. The discrepancy between these findings and the results of Fay & Delise (1973) might be due to the fact that they studied active shortening while the changes in bladder cell length were passive.

## 2.3 General morphological description of isolated strips used for mechanical experiments

The strips that are used for mechanical experiments in chapter 3 were blotted and weighed afterwards in order to determine their cross-sectional area. This made them unsuitable for histological study. For the latter purpose, strips composed mainly of longitudinal muscle were dissected out of rabbit bladders filled with 10 ml Krebs solution. Either the bladders were filled before dissection, or the strips were fixed at their in situ length.

In one series of 6 strips the preparations were fixed in glutaraldehyde embedded in plastic and cross-sectioned for phase-contrast microscopy. From photomicrographs the relative area of smooth muscle was measured to be  $67 \pm 3\%$ .

In another series of 6 strips the tissues were fixed in Bouin's solution and then paraffin-embedded. Cross sections and longitudinal sections  $7\text{ }\mu\text{m}$  thick were cut and stained with resorcin-fuchsin and picric acid - thiazin red for differentiation between smooth muscle elastic fibrils and collagen (Romeis 1968). The muscle bundles were found to be surrounded by loose connective tissue containing considerable amount of collagen. The relative cross-sectional area consisting of muscle was the same as for the series described above. Only a very limited number of elastic fibrils was seen among the muscle bundles. A high amount of elastic fibrils was found around intrasural vessels and in concomitantly embedded rat portal veins.

On electron-micrographs of both rabbit and guinea pig bladder collagen fibrils are seen surrounding the individual muscle cells. No further study regarding their relationship to the muscle cells was done (regarding cell-collagen relationship see Gabella 1977).

### 3 MECHANICAL PROPERTIES

#### 3.1 Introduction

Active force produced by a skeletal muscle increases with increasing extension of the muscle until a maximum is reached. Further stretch leads to a progressively decreasing active force. The mechanism behind this length-active tension relation was long obscure but certain features of it has now been adequately explained (Gordon et al 1966). The decrease in force seen when the muscle is stretched beyond the optimal length coincides with the decreasing overlap between the thick and thin myofilaments. Thus fewer cross bridges can participate in force production. The decrease in force at lengths shorter than optimal has been more difficult to explain. An increasing internal load could be of importance in this part of the length-tension diagram (Gordon et al 1966) but evidence has been presented that the electromechanical coupling especially in the central parts of the muscle cell becomes less efficient at shorter lengths (Rüdel & Taylor 1971).

Smooth muscle has a length-tension relation that in principle has the same features as that of skeletal muscle (see e.g. Spedden 1960, Csapo 1962, Stephens, Kroeger & Mehta 1969, Halpern, Mulvany & Warsaw 1978, paper I). An important difference is however that smooth muscle preparations can develop tension at much shorter length relative to the optimal than skeletal muscle. Section 3.3 will deal with the length-tension relation of rabbit bladder strips and possible explanations regarding the shape of it. Section 3.4 contains an attempt to determine the length-active tension relation of single guinea pig bladder cells.

The relationship between load and shortening velocity is an important characteristic of the contractile machinery. Hill (1938) in a classical series of experiments showed that shortening velocity ( $V$ ) can be described by a hyperbolic function of the force ( $P$ )

$$V(P) = a - b(P_0 - P) \quad \text{eqn. 1}$$

This equation represents a rectangular hyperbola with asymptotes at  $V = -b$  and  $P = \frac{a}{b}$  and a  $P$ -axis intercept at  $P = P_0$ , the isometric force. Until recently it was thought that the constant  $a$  reflected directly the energy liberated during shortening. Later investigations have cast doubts on this direct relation between thermal and mechanical events (see e.g. Simmons &

Jewell 1974) The force-velocity relation is however still of great importance as a means of characterizing properties of the contractile system

The muscle model suggested by Hill (1938) separates a contractile component and an undamped series elastic element. During an isometric contraction the contractile element was thought to shorten thereby stretching the series elastic element. A sudden change in the load of an isotonically shortening muscle was considered to cause an immediate adjustment of the length of the series elastic element and of the shortening velocity of the contractile component according to its force-velocity relation. Thus with this model no transient behaviour is to be expected. Experiments with this theoretical background has been performed on smooth muscle which has been found to behave qualitatively as other muscle types.  $V_{max}$  is however much lower than in skeletal muscle (see e.g. Stephens, Kroeger & Mehta 1969, Gordon & Siegelman 1971, Herlihy & Murphy 1974, Hellstrand & Johansson 1975).

During recent years the two-component model by Hill has been challenged mainly as a result of new studies on skeletal muscle. A major part of the elasticity of the actin muscle in well controlled studies is now considered not to be directly in series with the contractile machinery but to be associated with the cross-bridges themselves (Huxley & Simmons 1971, Ford, Huxley & Simmons 1977). Moreover the shortening velocity does not change instantaneously to a new steady level after a load change. The adjustment of the kinetics of the crossbridges takes a certain time (Civan & Podolsky 1966). The same is the case with regard to force following a length step (Huxley & Simmons 1971, Ford, Huxley & Simmons 1977). Velocity and force transients have recently been observed also in smooth muscle (Study III; Hellstrand & Johansson 1979; Mulvany 1979).

The mechanical characteristics of strips of longitudinal smooth muscle from rabbit urinary bladder at different lengths (3.6) and with different modes of activation (3.7, 3.8) have been studied using the isotonic quick release method devised by Jewell & Wilkie (1960) for skeletal muscle. The series elastic element, the force-velocity relation and the velocity transients will be characterized (3.5, 3.7). An analysis of the applicability of the quick release method for the present purpose will be given in the methods chapter.

## 3.2 Outline of methods

The following refers to the experiments described in sections 3.3, 3.5, 3.6, 3.7 and 3.8. The methods used in 3.4 will be described in that section.

Rabbits weighing between 2 and 3 kg were killed by a blow on the neck. The bladders were dissected out and transferred to oxygenated Krebs solution. Longitudinal strips containing principally longitudinal muscle (see 2.8) were dissected out from bladders filled with 10 ml Krebs solution. The in-situ length of the preparation at 10 ml will be referred to as  $L_{10}$ . This was generally about 5 mm. The preparation weight was from 0.5 to 4 mg. The strip was then mounted in an apparatus that could keep the muscle isometric and when desired release the muscle to previously determined afterloads. 3 types of apparatuses were used. For the determinations of length-tension relations in section 3.3 the apparatus described by Hellstrand, Johansson & Ringberg (1972) was used. The isotonic lever had an equivalent mass about 2.5 g. This made it unsuitable for the study of fast events but it was appropriate for the studies in 3.3. Part of the experiments in 3.3 and all experiments of 3.6 and 3.8 were performed with in an improved apparatus (regarding the design see: Johansson 1973). The equivalent mass of its lever was 150 mg and the compliance of the whole system was 10  $\mu\text{m/g}$ . The force and velocity output was recorded on a linear direct-writing oscillograph (Devices MX4). This apparatus gave adequate registrations from about 50 ms after the release. The period 0-50 ms was disturbed by oscillations originating mainly in the force transducer. To enable registration of the events immediately after a release a further improved apparatus was used. Its lever has an equivalent mass of 8 mg (for further details see Sjölin, Hellstrand & Clementz 1978, Hellstrand & Johansson 1979). The output of the force transducer and of the photo electric device registering lever movement are displayed on a storage oscilloscope (Tectronics 5301H) and recorded by a Grass kymograph camera. Simultaneous slow recordings on a linear direct-writing oscillograph (Devices MX4) were used to monitor the experiments. The signals were also stored on magnetic tape (Tandberg 100 or Thermionic Store 4 instrumentation recorder) for further analysis. This setup was used for sections 3.5 and 3.7.

The preparations were kept in a solution of the following composition in mM: NaCl 120, KCl 6.0,  $\text{MgCl}_2$  1.2,  $\text{CaCl}_2$  5, glucose 11.5 and tris(hydroxymethyl) aminomethane (Trizma Base, Sigma Chemical Co) 23. (It is referred to as N tris). The solution had previously been titrated with HCl at 37°C.

to pH 7.4. The osmolality measured by means of an Osmometer 31 LAS (Advanced Instruments Inc) was close to 300 mOsm. The solutions were continuously bubbled with 100 % O<sub>2</sub>. The preparations were allowed to accommodate for 1 h before the experiments were started. Two means of stimulation were used. K<sup>+</sup>-contractures were elicited by changing the H-tris solution to a solution (K-tris) in which all NaCl had been exchanged with equimolar amounts of KCl. The muscles could also be stimulated electrically with square waves or AC current through platinum electrodes.

Passive tension was 2-4 % of maximum active tension unless otherwise stated. Results are given as mean  $\pm$  SE.

According to Edean & Nilsson (1972) the degree of damping of the lever is critical in experiments on the mechanics of heart muscle due to effects of oscillations on the contractile function. If the lever was underdamped the inertial oscillations were found to deactivate the muscle and the shortening velocity was impaired. Reservations against the damped-release method has however been put forward (Gülich 1974). The releases in the present studies are all undamped. To find out whether no deactivating effects of inertial oscillations occur in bladder preparations experiments of the following design were made. Bladder strips are stimulated electrically. Early during the rising phase of one contraction the muscle was allowed to perform an afterloaded shortening. Later during another contraction the muscle was released to the previous afterload. If no inactivation occurred during the quick release the shortening velocity at a given degree of shortening should be the same for the afterloaded contraction (devoid of oscillations) and after the quick release (possessing oscillations). Fig 7 gives the results of one experiment. The two shortening curves superimpose almost during the first 75 ms after the quick release. During 0 - 75 ms shortening velocity is actually higher after the quick release. The obvious conclusion must be that inertial oscillations after quick releases have no deactivating effects on the smooth muscle preparation.

### 3.3 Length tension relations of bladder strips in vitro

Strips containing longitudinal smooth muscle from rabbit urinary bladder were used (1). After the accommodation period the strips relaxed in Ca free H-tris and were then transferred for 5 min to H-tris solution which elicited a contracture tension reaching a plateau level in 1-2 min. The bathing solution was then changed back to Ca<sup>2+</sup> free H-tris and the strips



$-\Delta L$   
mm  
0.2—

0.0—

P  
mN  
5—

0—

1

18

1

Fig. 7 An afterloaded isotonic contraction and a quick release to the same afterload of an electrically stimulated rabbit bladder strip (length 6.0 mm). In order to superimpose the length registrations the contraction ending with an afterloaded isotonic shortening is delayed in the figure relative to the quick release response. The length change in the quick release registration is typically an initial elastic recoil followed by an intermediate phase of rapid shortening and finally a slower shortening that can be superimposed upon the afterloaded shortening. The intermediate phase lasts about 75 ms.

rapidly relaxed. Time in this solution was 10 min. These steps were then repeated at different muscle lengths. The experiments generally started at  $L_{10}$  and the muscles were then shortened in steps. When enough data had been obtained the muscles were stretched in steps. After the experiments the strips were gently blotted and weighed. Their cross sectional area at the length where they developed maximum active tension was calculated assuming a density of  $1.0 \text{ mg/mm}^3$ . Fig. 8 shows the length-tension relations for 5 strips treated as just described. Active tension (total minus passive tension) starts to develop at a length of about 35 % of  $L_{10}$ . Active tension increases with stretch until a maximum is reached at a length of  $206 \pm 4 \%$ .

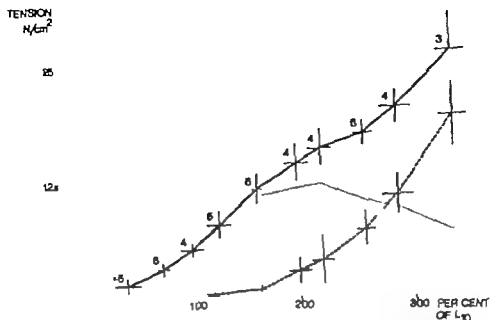


Fig. 8 Mean length-tension relations of 5 strips of longitudinal smooth muscle from rabbit urinary bladder.  $L_{10}$  is the length of the strip at a bladder volume of 10 ml. Relative lengths are divided into 10 classes. Continuous line Total tension. Broken line Passive tension. Dotted line Active tension. The bars indicate SD from 1.

of  $L_{10}$  (n = 5). Maximum active tension was  $12.5 \pm 0.4 \text{ N/cm}^2$ . Further stretch results in decreasing active and increasing passive tension. After correction for non-muscular tissue (see 2.8) mean active tension is  $19 \text{ N/cm}^2$ .

The shape of the length-tension curve obtained here is typical for mammalian smooth muscle (see e.g. Stephens, Kroeger & Mehta 1969; Lowy & Mulvany 1973). The bladder strips begin to develop tension at a length about 17% of the optimal. This agrees well with the 13–25% obtained by Stephens et al. (1969) and differs markedly from skeletal muscle which do not normally develop tension at lengths shorter than 65% of optimal (Gordon, Huxley & Julian 1966). The absence of Z-discs in mammalian smooth muscle might be of importance for their ability to shorten extensively. It could also be possible that the lesser efficiency of the electromechanical coupling at short muscle lengths reported by Rüdell & Taylor (1971) does not apply to smooth muscle but there is no evidence for this. As shown in chapter 2 no slippage between the muscle cells exist for a bladder radius interval covering the whole rising part of the length-tension curve. Slippage can

therefore be ruled out as a reason for the slow rise in active tension relative to increase in muscle length

Increasing the  $\text{Ca}^{2+}$  concentration in the K tris solution to 10 mM doubled the contractile force (1) but the shortest length at which active tension occurred was virtually unchanged. Shortening down to 17 % of optimal length thus probably represents the maximal isotonic response. Lowering of extracellular  $\text{Ca}^{2+}$  to  $\approx$  5 mM diminishes isometric force by a factor of ten compared to force output in 10 mM  $\text{Ca}^{2+}$ . The shortening capacity decreases however only slightly.

It has been found for skeletal muscle that the active tension developed at a certain length is the same irrespective of whether this length is reached passively or by isotonic shortening from a greater length (Gordon et al 1966). It was observed in the present studies (1) that this is not always the case for bladder strips. A series of 6 experiments was performed to compare isometric and isotonic length-tension relations. The strips were activated by K-tris following the same time schedule as described above and isometric tension was measured. At every second contraction the muscle was allowed to perform afterloaded isotonic shortenings and its length was varied as above. Up to initial lengths about 165 % of  $L_{10}$  the tension produced at a certain length was the same irrespective of how that length was reached. At lengths above 165 % of  $L_{10}$  the muscle could no longer shorten isotonicly to the point on the isometric length-tension curve corresponding to the afterload. This shortening defect which was most pronounced for intermediate and high afterloads increased progressively with further increases in length. At very small loads the shortening defect was almost absent. To see if the shortening defect could be due to e.g. overstretch of injured tissue in the knot region three experiments with the following design were performed. Three 0.1 x 0.5 mm silver markers were placed between the muscle bundles in the preparation thereby dividing it into four segments. Isometric and isotonic contractions were performed and the distances between the markers were measured with a dissection microscope. The inability of the muscles to shorten isotonicly from large starting lengths to corresponding points on the isometric length-tension curve was apparent and this shortening defect occurred in all four segments, i.e. also in the central ones which were unaffected by knot injuries.

Differences between isometric and isotonic length tension curves resembling those presented above have been noted also by workers using other smooth muscles e.g. Dobrin (1973) on dog carotid artery and Stephens & Van Niekirk (1976) on dog trachealis muscle. The phenomenon in these tissues seems to be at least partially reversible and differs in this respect from the irreversible changes described by Peterson & Paul (1974).

No satisfactory explanation for the shortening defect is available. It could be possible that cell slippage exists at extreme lengths but according to section 2.5 the relationship between bladder radius and cell length is linear up to 100 ml volume which corresponds to 215% of  $L_{10}$  and the shortening defect appears already at initial lengths from 165% of  $L_{10}$ . As the tension plateau during a K<sup>+</sup> contracture lasts for a considerable time the possibility of a decrease in activation with time as an explanation is unlikely.

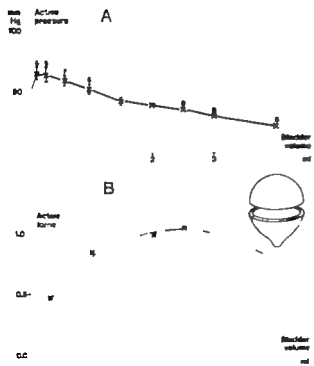
The shortening defect is probably of minor physiological importance as it appears only at lengths that correspond to bladder volumes near or above the normal maximum. Ruptures of the mucosa often occur in the rabbit bladder at 100 ml content volume (corresponding to 215% of  $L_{10}$ ).

### 3.4 Calculation of cellular length-active tension relations

The smallest functional component in skeletal muscle is the sarcomere. In smooth muscle it is not known whether a corresponding structural unit exists or not. At present the cell has to be considered the smallest functional part of smooth muscles. Isometric twitches have been elicited in isolated single cells from toad stomach (Fay et al 1976) and shortening velocity was recorded in such preparations by Bagby (1974). To my knowledge no length tension relations of single isolated cells have been reported.

Where a single isolated cell produces force only end-to-end it is possible that cells in situ transmit force over many parts of their surface to neighbouring cells. In this section I will try to calculate length-tension relations from mean guinea pig bladder cells in situ from volume-active pressure relations obtained in vivo. The experiments were carried out on ether-anesthetized guinea-pigs weighing about 400 g. The abdomen was opened and the bladder was cannulated in the mesentery. The mesentery was ligated and cut 0.5-1 cm from the bladder and sewed down together with surrounding tissue through plastic tubes with platinum electrodes at each

end. The electrodes were connected to Grass S44 stimulators via a Grass SIU5 isolation unit. The cannula was connected to a Statham P23 DC pressure transducer. Through a branch tube the bladder could be filled with Krebs solution to desired volumes, starting at zero. After the pressure had stabilized the bladder was stimulated for 15 s by a train of supramaximal square waves. Pressure increased rapidly to a maximum and then declined to some extent before the stimulation was ended. The bladder was filled to a new volume and the procedure repeated. When the highest desired volume was reached the bladder was emptied in steps and stimulated again. Time between stimulations was 10 min. Fig 9A summarizes the results. Maximum active pressure was obtained at 0.15 ml bladder volume and pressure in the relaxed bladder was negligible at all volumes. Assuming a spherical form which is

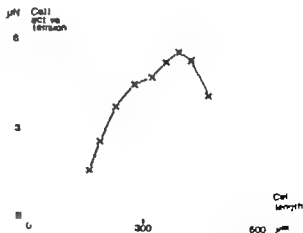


**Fig. 9** In vivo experiments with bilateral pelvic nerve stimulation Guinea-pig. **A:** Active pressure vs content volume. Four animals were used. Mean  $\pm$  SE. Numbers of determinations indicated in the figure. Maximum active pressure occurs at 0.15 ml. **B:** Force produced by the longitudinal muscle holding the two indicated hemispheres together vs bladder volume. Maximum force at 2.5 ml. From VI.

an appropriate approximation (see above) the volume-pressure relation can be transformed to a volume-force curve. The force trying to separate the two hemispheres in Fig 9B is  $F = r^2 \pi p$  ( $r$  is bladder radius  $p$  is pressure). This separating force is balanced by the force produced by the longitudinal smooth muscle  $F = A T$  ( $A$  = area of longitudinal muscle  $T$  is tension /unit of area/). The active force developed by the wall of the bladder reached a maximum of 1.04 N at 2.5 ml bladder volume (Fig 9B).

The total number of longitudinal muscle cells transected according to the bladder model in Fig 9B was calculated. Mean thickness of the longitudinal layer was measured on the sections used in 2.3. The thickness times the circumference gave the area of longitudinal muscle at the equatorial region. From Fig 5 the relevant cell packing densities could be obtained. The total number of cross-sectioned longitudinal cells at the equatorial region was  $185000 \pm 24000$  ( $n = 6$ ). Using this figure the force /cell could be calculated at all volumes in Fig 9. From Fig 3B the corresponding cell lengths could be obtained. Fig 10 shows the results of these calculations in the form of a cellular length-tension curve. A maximum active force of  $5.5 \mu\text{N}$  is developed at a cell length of  $400 \mu\text{m}$ . This maximal force per cell is comparable to those obtained by Fay (1977) in isolated toad stomach cells and by Pulvany & Halpern (1976) in vascular smooth muscle. If the length-active tension curve is extrapolated downwards by a straight line the length corresponding to zero tension becomes  $110 \mu\text{m}$  which is 28% of the optimal length. This is higher than the 17% obtained in 3.3 for whole

**Fig 10** Calculated length active tension relation for a average individual longitudinal smooth muscle cell of guinea-pig urinary bladder. From VI.



preparations of rabbit bladder strips but this difference may just reflect uncertainties in the calculations

The number of cells per  $\text{mm}^2$  at optimum length is 107000 according to Fig. 5. Active force per  $\text{cm}^2$  pure muscle bundle would then be 59 N. This value is high compared to most other vertebrate smooth muscles including those used for the results in section 3:3. One reason is probably that our in vivo experiments are performed on bladders with good metabolic conditions (no impairment of blood flow) and in contrast to strip preparations there are no injuries caused by knots or dissection procedures. Gabella (1976c) showed that guinea pig taenia coli strips produced more tension with carbachol than with high solutions or electrical field stimulation. He obtained  $42 \text{ N cm}^{-2}$  with carbachol corresponding to  $74 \text{ N cm}^{-2}$  when correction was made for nonmuscular tissue. Stimulation of the pelvic nerves to the bladders could on these grounds be expected to give a more powerful contraction than what is obtained by K. tris in 3:3.

The reason for the high active force in some mammalian smooth muscle is not known. Rosenbluth (1965) showed that the myofilaments of toad jejunum cells are not in parallel with the long axis of the cells. This could be a factor that increases cellular force. However, according to Gabella (1979c) myofilaments in taenia coli cells appear almost parallel to the long axis of the cells at all degrees of shortening. Myosin filaments are  $2 \mu\text{m}$  long in rabbit portal vein (Ashton, Soaiyo & Soaiyo 1975) compared to  $1.6 \mu\text{m}$  for skeletal muscle (Page & Huxley 1963). According to Riegger (1971) active force in muscle is directly proportional to thick filament or sarcomere length. Longer thick filaments enable more cross-bridges to work in parallel, thereby increasing force output. Maximum active force for mammalian smooth muscle is about  $30 \text{ N/cm}^2$  (Close 1973). If everything else was constant,  $41 \text{ N/cm}^2$  ( $30 \times 2.2/1.6 \text{ N/cm}^2$ ) would be expected in skeletal muscle if the thick filaments had the same length as in smooth muscle. The ratio of thick filament length can thus not account for the whole difference. The significance of the high actin and low myosin content in smooth compared to skeletal muscle (Murphy et al 1974) remains to be assessed. Smooth muscle has lower tension cost than skeletal muscle (Paul et al 1976, Hellstrand 1977, Johansson 1978) suggesting that the duration of the cross-bridge cycle is longer. If this was due to a longer relative time at the attached state than in skeletal muscle, this would give a high force output.

### 3.5 Isotonic behaviour after quick release

In Fig 7 a typical quick release is shown. The behaviour after the release can be divided into 3 parts. There is an immediate recoil followed by a phase of fast shortening lasting 50 - 75 ms. Finally the shortening slows down and superimposes almost exactly on a corresponding afterloaded isotonic shortening curve. This section will deal with the three phases of the quick release response in the order that they appear.

The immediate recoil has usually been attributed to the series elastic element according to Hill's (1938) two-component model (see e.g. Jewell & Wilkie 1958, Edman & Nilsson 1972, Hellstrand & Johansson 1975). In smooth muscle the recoil of this series elastic (SE) element when the muscle is released to zero tension varies considerably. The earlier the study the more compliant is the SE element found to be [Åberg (1967) 13% guinea-pig taenia coli; Stephens & Kromer (1971) 7.5% dog trachealis muscle; Neiss (1978) 4% rabbit mesotubarium]. This may partly be due to different preparations and methods in the different studies. Fig 11 shows SE

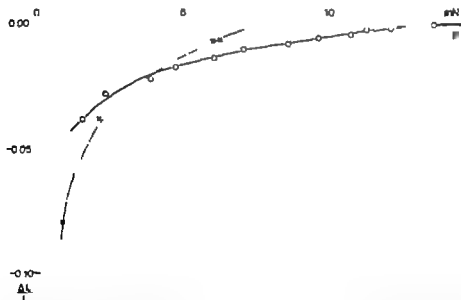


Fig. 11 Series elastic recoil of a rabbit bladder strip and a rat portal vein. Open circles: Bladder (length 4.76 mm, isometric force 3.0 N/cm<sup>2</sup>). Crosses: Portal vein (length 3.00 mm, isometric force 1.7 N/cm<sup>2</sup>). Fits to eqn 2 are shown by full and broken lines respectively. Constant  $k$  is 62 for bladder and 36 for portal vein, indicating that the series elastic element is more compliant in the latter. AC stimulation.



load extension-curves in a rat portal vein and a rabbit bladder preparation. Despite the fact that the same apparatus and the same mode of stimulation were used there is a considerable difference in SE recoil between the two muscles. The experimental data could be fitted to an exponential equation of the following form

$$F = A \exp(k \Delta L / L) + B \quad (\text{eqn 2})$$

The stiffness ( $\frac{\Delta P}{\Delta L}$ ) increases in proportion to force with the proportionality constant  $k$ . In paper III  $k$  was  $91 \pm 13$  ( $n = 7$ ) for urinary bladder and  $28 \pm 5$  ( $n = 5$ ) for portal vein. In paper V  $k$  was  $53 \pm 6$  and  $34 \pm 4$  respectively. The difference in urinary bladder stiffness between the two studies may be related to differences in preparation length and active tension development. It is not known which structures that are responsible for the SE recoil. In skeletal muscle mounted under strictly controlled conditions the recoil is considered to be due to elastic behaviour of the cross-bridges themselves (e.g. Huxley & Simmons 1971). The SE recoil in the bladder muscle is however several times larger than in skeletal muscle (regarding the latter see e.g. Bressler & Clinch 1974, Ford, Huxley & Simmons 1977). It is therefore unlikely that a major part of the series elasticity would reside in cross bridges in bladder muscle but it has been suggested that a small fraction may reside in these structures (Hellstrand & Johansson 1979). Meiss (1978) calculated the extension of the SE element for one isolated toad stomach muscle cell in the study by Fay (1977) and obtained a value 2.2% of the cell length. This is only somewhat smaller than the 3.4% maximal SE recoil in bladder muscle found in study III. This comparison could indicate that also in bladder muscle a major portion of the series elastic element resides in structures on or in the cell. At present no clearcut explanation for the difference between bladder and portal vein exists. A difference in cell size could play a role but the cell packing density in rat portal vein ( $66000 \text{ mm}^{-2}$  Uvelius unpublished) is not very different from what is found in bladder (Fig. 5). Another possibility is that extracellular structures are of importance. One difference observed between the two muscle types in the present studies is the richer amount of elastic fibrils in portal vein. This could explain not only the high passive tension in portal vein but perhaps also part of the high compliance of its SE element.

The intermediate phase of the quick release response characterized by fast shortening was first investigated in (III). It has since been described and analyzed by Hellstrand & Johansson (1979) and Hellstrand (1979). It seems possible that it is an effect of cross-bridge rearrangement and change of kinetics after the release. This transient phase subsides after about 75 ms after the release (III).

Determinations of shortening velocity for force-velocity curves were performed at 100 ms after release thus avoiding effects of the transient. As shown in Fig. 12 the experimentally determined force-velocity points can be fitted to the Hill equation. The curve-fitting was performed either by using a linearized form of Hill's equation (II) or by a computer program that took into account errors in both coordinates (III-IV-V). As will be seen in the following sections  $V_{max}$  varies with muscle length and mode of stimulation.

### 3.6 Influence of muscle length on force-velocity relations

In this section  $K^+$  activated rabbit bladder strips were used (see II).  $V_{max}$  was  $29 \pm 0.13$  l/s ( $n = 8$ ) at  $L_{10}$ . When the muscle was stretched  $P_0$  (the isometric tension) increased according to the length-active tension relation of the muscle (see 3.3).  $V_{max}$  was also found to be length-dependent even when it was expressed in relation to actual muscle length. At  $L_{10}$   $a/P_0$  was  $0.17 \pm 0.02$  ( $n = 8$ ) and  $b$  was  $0.052 \pm 0.008$  muscle lengths per second ( $n = 8$ ). These values are of the same order of magnitude as those found for instance by Mashima & Huxley (1969), Stephens et al. (1969) and Merlihy & Murphy (1974). As  $a$  and  $b$  no longer have any certain relation to the real events in muscle there is no point in discussing the small differences that exist. Within the interval  $0.69 L_{10} - 1.44 L_{10}$   $b$  was constant whereas  $a/P_0$  varied in relation to  $P_0$  indicating that  $a$  was length independent over this interval. The results correspond well with those of Gordon & Slegman (1971) who also found  $a$  and  $b$  to be constant although the length interval was smaller in their study.

Hill's equation can be rewritten as  $V = [b/(P - a)](P_0 - P)$ . With constant values for  $b$  and  $a$  and with  $P$  (the afterload) kept unchanged in the experiment  $V$  should vary in proportion to  $(P_0 - P)$ . Releases to small afterloads were performed in a separate study. The results indicated that there is a linear relationship between  $P_0$  and  $V$  at lengths up to about  $1.25 L_{10}$ . This means that up to  $1.25 L_{10}$  the length-active force relation and the

length -  $V_{max}$  curve vary concomitantly with muscle length. At lengths greater than this shortening velocity increases more than  $(P_0 - P)$ . The reason for this is not clear. There might be a length-effect on the parameters  $a$  and  $b$  at these degrees of stretch. It is also possible that the force carried by the contractile units is less than  $P$  due to an unloading effect of the passive parallel elasticity which begins to carry force at these lengths.

### 3.7 Effects of different modes of activation on contraction dynamics

In sections 3.5 and 3.6 it was shown that Hill's equation fits well with observed shortening velocities against different loads. When  $P_0$  was altered by a change in muscle length  $V_{max}$  was affected to almost the same proportion. This could lead to the assumption that there exists a unique relationship between  $P_0$  and  $V_{max}$ . It has been suggested however that  $V_{max}$  in smooth muscle may vary with the mode of stimulation being higher during activation by AC current than during  $K^+$ -contractures (Hardung & Laslett 1966, Hellstrand & Johansson 1975, Murphy 1976). On the other hand  $P_0$  seems also to be higher with AC stimulation (Gabella 1976c). It would be of great interest if it could be shown conclusively that  $V_{max}$  is affected differently than  $P_0$  with different modes of stimulation (AC current,  $K^+$ ) since this finding would indicate that the kinetics of the contractile process is different in tonic ( $K^+$ ) and phasic (AC) activity. The problem has not been studied systematically so far.

In (V) 2-3 min long  $K^+$ -contractures are elicited in rabbit bladder strips. At the peak of each contracture a quick release was made using different afterloads. After a sufficient number of such responses had been recorded the muscle was kept in  $K^+$ -tris and repeatedly stimulated by AC current (25-100 Hz, 2-4 V, duration 2-5 s) at 1/2 - 1 min interval. Supramaximal AC stimulation usually gave a higher isometric tension than obtained in the  $K^+$  contractures (cf. Gabella 1976c). Therefore the intensity of the AC stimulation was adjusted to give the same  $P_0$  as the preceding  $K^+$  contractures.

The initial (SE)  $\tau$  values at different afterloads were fitted by a computer program to eqn. 2 (see above). The characteristics of the elastic element were found to be unaffected by mode of stimulation: the constant  $k$  of eqn. 2 was  $51 \pm 6$  (n = 6) for AC stimulations and  $54 \pm 3$  (n = 6) for  $K^+$  contractures. Hellstrand & Johansson (1979) found that the series elasticity was

unaffected by changes in temperature of the bathing medium. This together with the apparent lack of influence of stimulation mode (as contrasted to the marked effects on  $V_{\max}$  see below) suggests that a major part of the SE recoil is a passive phenomenon.

The length response after the elastic recoil was fitted by a computer program to a double-exponential model (Hellstrand & Johansson 1979) of the form

$$L(t) = L + A_1[1 - \exp(-t/\tau_1)] - A_2[1 - \exp(-t/\tau_2)] \quad \text{eqn 3}$$

The amplitude ( $A_1$ ) of the fast shortening component was unaffected by mode of stimulation (paper V). The rate constant ( $1/\tau_1$ ) was however about 25% smaller in  $K^+$ -contractures. A comparable effect is seen when bladder muscles are cooled (Hellstrand & Johansson 1979). Their suggestion that the fast shortening component is not a passive phenomenon is further validated by the present results. The fast exponential component subsides rapidly and is practically over at 100 ms after the release, the time at which shortening velocity was measured. The force-velocity values were fitted by a computer program to eqn 1 in 3.1.  $V_{\max}$  was found to be about 30% lower in  $K^+$ -contractures than in responses to AC stimulation ( $0.26 \pm 0.03$  (n = 6) l/s vs  $0.37 \pm 0.05$  l/s). Force-velocity relations from a typical muscle is shown in Fig. 12.

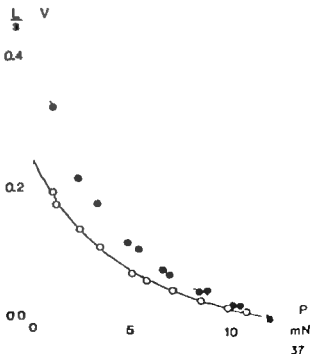


Fig. 12. Force-velocity relations of a bladder strip. Velocity is measured 100 ms after the release. Filled circles: AC-stimulation; open circles:  $K^+$ -activation. Fits to Hill's eqn are shown by broken and full lines respectively. From V.

length -  $V_{\max}$  curve vary concomitantly with muscle length. At lengths greater than this shortening velocity increases more than  $(P_0 - P)$ . The reason for this is not clear. There might be a length-effect on the parameters  $a$  and  $b$  at these degrees of stretch. It is also possible that the force carried by the contractile units is less than  $\frac{1}{2} P_0$  due to an unloading effect of the passive parallel elasticity which begins to carry force at these lengths.

### 3.7 Effects of different modes of activation on contraction dynamics

In sections 3.5 and 3.6 it was shown that Hill's equation fits well with observed shortening velocities against different loads. When  $P_0$  was altered by a change in muscle length  $V_{\max}$  was affected to almost the same proportion. This could lead to the assumption that there exists a unique relationship between  $P_0$  and  $V_{\max}$ . It has been suggested, however, that  $V_{\max}$  in smooth muscle may vary with the mode of stimulation being higher during activation by AC current than during  $K^+$ -contractures (Hardung & Laszt 1966, Hellstrand & Johansson 1975, Murphy 1976). On the other hand  $P_0$  seems also to be higher with AC stimulation (Gabella 1976c). It would be of great interest if it could be shown conclusively that  $V_{\max}$  is affected differently than  $P_0$  with different modes of stimulation (AC current  $K^+$ -trials) since this finding would indicate that the kinetics of the contractile process is different in tonic ( $K^+$ ) and phasic (AC) activity. The problem has not been studied systematically so far.

In (V) 2-3 min long  $K^+$ -contractures were elicited in rabbit bladder strips. At the peak of each contracture a quick release was made using different afterloads. After a sufficient number of such responses had been recorded the muscle strips were kept in  $K^+$  trials and repeatedly stimulated by AC current (25-100 Hz, 2-4 V, duration 2-5 s) at 1/2 - 1 min intervals. Supramaximal AC-stimulation usually gave a higher isometric tension than obtained in the  $K^+$ -contractures (cf. Gabella 1976c). The afterload of the AC stimulation was adjusted to give the same  $P_0$  as the preceding  $K^+$  contractures.

The initial (SE) recoils at different afterloads were fitted by a computer program to eqn. 2 (see above). The characteristics of the elastic element were found to be unaffected by mode of stimulation; the constant  $k$  of eqn. 3 was  $51.6$  (n = 6) for AC stimulations and  $54.3$  (n = 6) for  $K^+$  contractures. Hellstrand & Johansson (1979) found that the series elasticity was

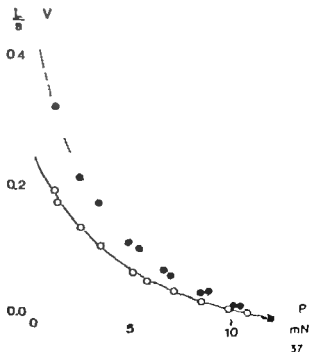
unaffected by changes in temperature of the bathing medium. This together with the apparent lack of influence of stimulation mode (as contrasted to the marked effects on  $V_{max}$  see below) suggests that a major part of the SE recoil is a passive phenomenon.

The length response after the elastic recoil was fitted by a computer program to a double-exponential model (Hellstrand & Johansson 1979) of the form

$$L(t) = L_0 + A_1[1 - \exp(-t/\tau_1)] + A_2[1 - \exp(-t/\tau_2)] \quad \text{eqn 3}$$

The amplitude ( $A_1$ ) of the fast shortening component was unaffected by mode of stimulation (paper V). The rate constant ( $1/\tau_1$ ) was however about 25% smaller in  $K^+$  contractures. A comparable effect is seen when bladder muscles are cooled (Hellstrand & Johansson 1979). Their suggestion that the fast shortening component is not a passive phenomenon is further validated by the present results. The fast exponential component subsides rapidly and is practically over at 100 ms after the release, the time at which shortening velocity was measured. The force-velocity data were fitted by a computer program to eqn 1 in 3.1.  $V_{max}$  was found to be about 30% lower in  $K^+$  contractures than in responses to AC stimulation ( $0.26 \pm 0.03$  vs  $0.37 \pm 0.05$  l/s). Force-velocity relations from a typical muscle is shown in Fig. 12.

Fig. 12. Force-velocity relations of a bladder strip. Velocities measured 100 ms after the release. Filled circles: AC stimulation; open circles:  $K^+$  activation. Fits to Hill's eqn. are shown by broken and full lines respectively. From V.



There is no straight forward explanation for the fact that  $V_{max}$  is lower in  $K^+$ -contractures than in AC-contractions of equal  $P_0$ . It has been reported by Jones et al (1973) that vascular smooth muscle cells swell and that their thick myofilaments disrupt when the vessels are placed in KCl high media. These authors suggested however that the disruption of the thick filament lattice took place during the fixation of the tissue for electron microscopy. They base this suggestion on the fact that the tension output was the same as for  $K_2SO_4$  solutions which did not cause swelling of the cells. The difference in shortening velocity described above could be an effect of filament disruption if this change occurs also in the living muscle.

Another factor which could contribute to the different  $V_{max}$  values is the time the muscle is activated before the releases are made. The AC contractions are short-lasting and the releases are performed after  $\approx 5$  s whereas 1-2 min elapse before a  $K^+$ -contracture reaches its maximum.  $K^+$  activated slow fibres from *Xenopus laevis* shorten more slowly if a release is made late during a contraction compared with an early release (Lännergren 1978). The finding in paper IV that  $V_{max}$  declines faster than  $P_0$  during a single twitch might also be of relevance. Pelzer (1979) found that the rate of tension recovery in portal vein after vibration-induced relaxation is slower late during a K contracture. Altogether these results might indicate that the rate of the bridge-cycling is slowed down with time during prolonged contractions. The findings of Arner & Hellstrand (1980) that in relation to tension output the oxygen consumption decreases with time during  $K^+$ -contractures further support this idea. A possible explanation could be that an internal load develops during long-lasting contractions e.g. due to rigor complexes (Weber & Murray 1973) caused by insufficient ATP supply.

The  $V_{max}$  value for rabbit bladder muscle (0.37 l/s V) is high compared to many other smooth muscles but much lower than in skeletal muscle (from 7 l/s; Close 1972). The possible relationship between  $V_{max}$  and metabolic rate in smooth muscle is discussed by Hellstrand (1977).

### 3.8 Single twitch: Shortening velocity and active force

Paper IV gives an account on how  $P_0$  and  $V_{max}$  are affected in rabbit bladder at 30°C during single twitches elicited by single supramaximal square wave pulses.  $P_0$  and  $V_{max}$  were determined as the intercepts with the

force and velocity axis respectively of the computer fits of the data to Hill's equation

The time from stimulation to peak of the twitch was about 2 s.  $V_{\max}$  and  $P_0$  reached their maximum values at the same time about 1 s after stimulation.  $V_{\max}$  then declined rapidly. At the peak of the isometric force for instance the  $V_{\max}$  value was only 70% of its maximum. The  $P_0$  value on the other hand decreased more slowly and is at the peak of the isometric contraction only slightly less than maximum. A comparison of force-velocity curves from the rise and from the peak of the isometric response shows that they differ in the same manner as the curves obtained during AC and K stimulations respectively in 3.7. The possibility that inhomogeneity of activation could be of importance for the change in the force-velocity relation during the twitch was ruled out. Strips were marked with charcoal grains and were photographed while contracting. Only minor movements of the grains were observed.

The difference in time between the releases during the rise and those at peak of contraction is smaller than the difference between releases performed during AC and K contractions. They strengthen however the suggestion in 3.7 that the duration of activation is of importance for  $V_{\max}$  rather than the mode of stimulation.



#### 4 EFFECTS OF PROLONGED BLADDER DISTENSION ON LENGTH TENSION RELATIONS

In response to an increased functional demand smooth muscle will hypertrophy (see e.g. Gabella 1975). The development of the hypertrophy is fast as seen e.g. in rat portal vein subjected to increased intramural pressure (Johansson 1976). The cross sectional area of longitudinal muscle in this vessel has increased considerably within 8-11 days after the pressure rise. This fast hypertrophy shows that there exists an intimate dynamic relation between muscle cell size and the functional demands.

Peterson et al (1974) have induced hypertrophy in rat urinary bladder by injection of paraffine into the lumen. In the present study this method was used on female guinea-pigs weighing 550 to 600 g. Paraffine with a temperature just above melting point (43°C) was injected through a urethral cannula into the bladder of nembutal anaesthetized animals. Each animal had a matched control with the same body weight. Terramycin was added to the drinking water in order to avoid bladder infection. Despite this precaution half of the paraffine-injected animals had to be discarded due to loss of weight, probably as a result of bladder infection.

After 10 days the animal was killed and the bladder was emptied of urine leaving only the paraffine mass. A longitudinal bladder strip was marked out, measured, dissected free and mounted in an apparatus that could record isometric tension and isotonic shortening (lever equivalent weight 300 mg; see 3.2). The strip was stimulated by K<sup>+</sup> tris solution following the same time schedule as in 3.3. The minimum length to which the muscle could shorten actively when unloaded, as determined as was the length at which maximum active tension developed. These lengths will be expressed in relation to the in situ length of the strip. After the experiments the strip was blotted and weighed. The bladder was weighed before and after 3 days in 70°C 1 order to determine the water content of the tissue. The volume of the paraffine mass was measured. The control bladder was filled with Krebs solution to give a content volume equal to the paraffine volume. A strip was then marked out and treated as described above.

Table 1 gives the results from the 4 hypertrophic bladders and from their controls. The bladder weight increased from 0.43 ± 0.03 g in the controls to 1.30 ± 0.17 g. The control bladders weighed slightly more than guinea-pig bladders in chapter 2 (0.3 g) probably because the animals used here were

larger. There is little inflammatory reaction in the bladders of these selected animals. The increase in bladder weight is therefore not due to inflammatory oedema. The water content did increase however from  $78 \pm 0.9\%$  in the controls to  $86 \pm 0.3\%$  in the hypertrophic bladder. The study did not give any indication regarding structural localization of the increase in water content.

There was no significant difference in optimum length for force generation but this length is difficult to determine due to the broad and flat length-tension curve. The tension output for both controls and hypertrophic bladders in Table I was low compared to those in 3.3 and 3.4. The main reason is probably that the present strips contained all layers (mucosa to peritoneum) and no correction for the amount of non-muscular tissue has been made. The active tension per unit area was considerably lower in hypertrophic bladder ( $0.91 \pm 0.23$  vs  $1.67 \pm 0.15$  N/cm<sup>2</sup>). The same qualitative difference has been described by Johansson (1975) for portal vein.

Table I A comparison between hypertrophic (H) bladders and their controls (C). Content volumes between 1.2 and 2.1 cl. The length ( $L_{max}$ ) at which maximum active tension ( $P_{max}$ ) is developed is expressed in relation to the in situ length of the strip. Passive and active tension at  $L_{max}$  are expressed in relation to both wet weight/ $L_{max}$  and dry weight/ $L_{max}$ . The length ( $L_0$ ) that the unloaded muscle can shorten to is given in relation to the in situ length. The results were analyzed according to student's t-test for paired data.  $p < 0.05$  \*\* =  $p < 0.01$  \*\*\* =  $p < 0.001$

	C	H
bladder wet weight (g)	$0.43 \pm 0.03$	$1.30 \pm 0.17$
bladder dry weight (g)	$0.09 \pm 0.01$	$0.19 \pm 0.02$
water content (%)	$78 \pm 0.9$	$86 \pm 0.3$
$L_{max}/L_{in situ}$ (%)	$136 \pm 15$	$133 \pm 10$
$P_{passive}$ at $L_{max}$ wet (N/cm <sup>2</sup> )	$0.62 \pm 0.19$	$0.42 \pm 0.17$
$P_{passive}$ at $L_{max}$ dry (N/cm <sup>2</sup> )	$2.89 \pm 0.95$	$2.99 \pm 1.4$
$P_{max}$ wet (N/cm <sup>2</sup> )	$1.67 \pm 0.15$	$0.91 \pm 0.23$
$P_{max}$ dry (N/cm <sup>2</sup> )	$7.74 \pm 0.85$	$6.6 \pm 1.55$
$L_0/L_{in situ}$ (%)	$60 \pm 7$	$80 \pm 8$

and Gabella (1979b) for intestinal muscle  $P_{\max}$  per  $\text{cm}^2$  dry muscle would be  $7.7 \pm 0.9 \text{ N/cm}^2$  for the controls and  $6.3 \pm 1.6 \text{ N/cm}^2$  for hypertrophic bladders. These values did not differ significantly and might indicate that the decreased tension per unit area seen in hypertrophic bladders is due to water accumulation. Gabella (1979b) observed a considerable increase in intermediate filaments and a relative decrease in myofilaments and he suggested that this might be a factor contributing to the relative reduction in contractile force. He also suggested that the changes in intercellular collagen that he describes might be of importance. In the present study passive tension at  $L_{\max}$  was higher for control bladders ( $0.62 \pm 0.19$  vs  $0.42 \pm 0.17 \text{ N/cm}^2$ ) but if passive tension is considered in relation to dry cross-sectional area the difference disappears. This could indicate that the structures responsible for passive tension are not considerably changed in hypertrophic bladders.

Unloaded hypertrophic strips can shorten less relative to their in situ lengths than their matched controls (Table I). This indicates that the relation between the muscle cells or the properties of the contractile proteins within the cells have changed. According to Gabella (1979a) cell length is unchanged in intestinal muscle 3.5 weeks after onset of hypertrophy. Shortening capacity was not determined in that report and the time before sacrifice was longer than in this study. If in spite of this his results are applicable to the present study they might indicate that the properties of the myofilaments have altered. Comparisons of characteristics of the force-velocity relations between controls and hypertrophic bladders would obviously be of great interest. Such studies have not yet been performed. It is to be noted however that  $V_{\max}$  is lower in papillary muscles from hypertrophic hearts compared to controls (Hamrell & Alpert 1976).

## 5 GENERAL DISCUSSION

This discussion can be separated into 4 parts 1 The relation between preparation length (or bladder distension) and muscle cell length 2 the effect of preparation length on tension development (length-tension relations) 3 the isotonic behaviour of bladder muscle at different lengths and stimulation modes and 4 effects on length-tension relations induced by bladder hypertrophy

The cell lengths were measured in papers I and VI using two different methods The direct relationship seen between preparation length (or bladder radius) and cell length strongly suggests that the cells are coupled in series This is in accordance with the results from pig carotid artery by Murphy Driske & Cohen (1977) No slippage between the muscle cells could be detected even at considerable stretch Evidently results regarding tension output and shortening velocity vs e.g. preparation length represent in a direct way the behaviour of the individual muscle cells

If there exists a linear relationship between cell length and number of cells per unit cross sectional area it seems reasonable to assume that cell volume is independent of the degree of passive stretch Fay & Delise (1973) found that the stereometrically determined volumes of isolated muscle cells from toad stomach decreased up to 30% when the cells were stimulated and actively shortened The difference in results could indicate that whereas passive length changes do not induce net water movement over the cell membrane active shortening does

The length-tension relation of bladder muscle has the same general configuration as that of other smooth muscles (for ref. see Murphy 1976) Rabbit bladder strips can shorten to 17% of the optimal length for active tension development The low active tension when a bladder strip is stretched is as mentioned above not due to cell slippage As the cells are coupled in series the same length-tension behaviour is to be expected for individual cells The study includes an attempt to determine length-tension relations for single cells in intact guinea-pig bladders (VI) Maximum active tension (5.5  $\mu$ N per cell) is attained at a cell length of 400  $\mu$ m This active force corresponds to 59 N per  $\text{cm}^2$  a high value compared to 19 N per  $\text{cm}^2$  obtained for rabbit bladder strips It appears unlikely that this difference would be due to the different species used It is probably so that the intact guinea pig bladder t

has a better supply of oxygen and nutrients and that there is not tissue damage of the kind expected to occur when strips are dissected out. These favourable conditions do not apply to the isolated rabbit strips.

The capacity of smooth muscle to shorten extensively in comparison to skeletal muscle could be due to the absence of Z-discs and/or to better electromechanical coupling at shorter lengths. Variations in extracellular  $\text{Ca}^{2+}$  greatly influences force output in bladder muscle but the capacity to shorten is not much changed. Active force per unit cross sectional area in guinea-pig bladder muscle is higher than in skeletal muscle. Part of this might be explained by longer thick myofilaments as described in other smooth muscles (2  $\mu\text{m}$  vs 1.6 Ashton et al 1975). The cell length determinations in the present study (I-VI) show that parallel coupling which would increase tension output is virtually absent. The lower tension cost observed for some smooth muscles (Paul et al 1976, Hellstrand 1977) might indicate that in these muscles the crossbridges spend a longer time attached during a cycle than in skeletal muscle.

Hill's muscle model consisting of a contractile element (CE) in series with a series elastic element (SE) has been applied to smooth muscle by many investigators (e.g. Stephens, Kroeger & Hehta 1969, Gordon & Siegesan 1971, Johansson 1973, Hellstrand & Johansson 1975). The length of the SE and the shortening velocity of the CE depend on the load. According to the classical concept a change in load should instantaneously change SE length and CE shortening velocity. In skeletal muscle this model is an oversimplification as it has been shown that in well controlled studies (e.g. Ford, Huxley & Simmons 1977) a major part of the series elasticity is associated with the cross bridges. Moreover a change in load does not instantaneously change shortening velocity to a new steady level, a transitional phase exists (Civan & Podolsky 1966). Also in smooth muscle research the simple Hill model has been reconsidered. The change in shortening velocity following load change is not instantaneous (III Hellstrand & Johansson 1979). An intermediate phase exists characterized by fast shortening. Not until about 75 ms after the quick release is the length record superimposable on afterloaded isometric shortening with the same load. The intermediate phase lasts for a long time than the one described for skeletal muscle. It seems to be associated with cross bridge events as different modes of a tetanic (K $\rightarrow$ AC stimulation) affect  $V_{\text{max}}$  and the

rate constant of this transient to about the same extent ( $V$ ). It has also been shown that  $V_{max}$  and the rate constant both decrease when temperature is lowered (Hellstrand & Johansson 1979).

The force-velocity relation for bladder muscle is well approximated by Hill's equation.  $V_{max}$  increases with increasing muscle length to the same extent as isometric force ( $P_0$ ) in a major part of the rising phase of the length-tension relation. At lengths near those for optimal force,  $V_{max}$  increases more than active  $P_0$ . This might indicate that the dynamic constants in Hill's equation change, but it is equally possible that the force carried by the contractile machinery becomes smaller due to the increasing passive tension at these lengths.

$V_{max}$  is lower for K<sup>+</sup> contractions than for AC stimulations giving the same active force (paper V). The reason for the lower  $V_{max}$  for tonically contracting muscle is not known. In *Xenopus* slow fibres (Lennerngren 1978) shortening velocity to a given load decreases with time in K<sup>+</sup> high media. The reason for the lower  $V_{max}$  in the tonic contractions is not known, but it is possible that the myofilament lattice may be affected in KCl (cf Jones, Somlyo & Somlyo 1973) or that biochemical changes in the cell (e.g. change in ATP) may influence cross-bridge cycling rate. The SE behaviour is unaffected by mode of stimulation ( $V$ ) suggesting that its major part is passive. A minor part of the SE in bladder muscle has been suggested to reside in the contractile proteins or structures associated with them (Hellstrand & Johansson 1979; Hellstrand 1979).

Strips from bladders that are hypertrophied due to prolonged distension have the same optimum length for force development as their controls after normalization to the in-situ length. Maximum force is lower in hypertrophied muscle, but if correction is made for the increased amount of tissue, this difference disappears. The hypertrophied muscle cannot shorten to the same extent when unloaded as the controls. It is not known if this is due to changes in relations between the cells or to changes within the cells. The latter possibility seems the most probable, since cell length in intestinal muscle does not change with hypertrophy (Gäbel 1979a).

## 6 SUMMARY

Mechanical characteristics of smooth muscle from rabbit and guinea-pig urinary bladder were examined in relation to bladder morphology. Muscle cell length (determined by nitric acid maceration or morphometry on fixed tissue) was directly proportional to bladder radius (or stretch of isolated bladder strips) indicating a series coupling of the cells. The number of cross sectioned cells per  $\text{mm}^2$  varied between 35000 and 170000 and was found to increase in proportion to bladder radius indicating that cell volume is not effected by passive length changes. Length-tension (L-T) relations were determined for rabbit bladder strips. Active force was  $19 \text{ N/cm}^2$  at optimum length. The strips could shorten actively to 17% of this length when unloaded. During the major portion of the rising part of the L-T curve isometric and isotonic L-T relations were identical. At greater lengths the strips failed to shorten to the same length as when contracting from a shorter starting length. L-T relations for a single pig bladder cell were calculated. Max active tension at optimum length ( $400 \mu\text{m}$ ) was  $5.5 \mu\text{N/cell}$  corresponding to  $59 \text{ N/cm}^2$  pure muscle. Isotonic quick releases were performed on rabbit bladder strips. The immediate series elastic (SE) recoil was followed by a transient phase (75 ms) of rapid shortening and finally a steady shortening. Max SE recoil was 4%. SE characteristics were unaffected by mode of stimulation suggesting that it is predominantly passive.  $V_{\text{max}}$  was  $0.37 \text{ l/s}$  and  $0.26 \text{ l/s}$  when strips were activated by AC and  $\text{K}^+$  high media respectively. The rate constant for the transient was lower for  $\text{K}^+$  activation than for AC indicating that it is due to events associated with the contractile proteins. Hypertrophic bladder muscle had lower max active tension than controls but the difference disappeared after correction for higher water content in the former. Hypertrophic bladders could not shorten to the same ultimate length when unloaded as their controls.

## ACKNOWLEDGEMENTS

The study is supported by the Swedish Medical Research Council (04x-00028) the Medical Faculty University of Lund Svenska Sällskapet för Medicinsk Forskning and AB Hassle Göteborg.

## REFERENCES

- ÅBERG A 1967 Series elasticity of active taenia coli in vitro Acta Physiol Scand 69 348-354
- ÅBERG A K G & AXELSSON J 1965 Some mechanical aspects of an intestinal smooth muscle Acta Physiol Scand 64: 15-27
- AMOPOLSKY D 1928 Cyclic changes in the size of muscle fibers of the fallopian tube of the sow Amer J Anat 40: 459-468
- ARNER A & HELLSTRAND P 1980 Contraction of the rat portal vein in hypertonic and isotonic medium Rates of metabolism Accepted for publication in Acta Physiol Scand
- ASHTON F T SOMLYO A V & SOMLYO A P 1975 The contractile apparatus of vascular smooth muscle: intermediate high voltage stereo electron microscopy J Molecul Biol 98: 17-29
- BAGBY R M 1974 Time course of isotonic contraction in single cells and muscle strips from Bufo marinus stomach Amer J Physiol 227: 789-793
- BENNET M R & ROGERS D C 1967 A study of the innervation of the taenia coli J Cell Biol 33 573-596
- BURNSTOCK G 1970 Structure of smooth muscle and its innervation In Smooth muscle (Ed M Bulbring et al) pp 1-69 E Arnold Ltd London
- CIVAN M R & PODOLSKY R J 1966 Contraction kinetics of striated muscle fibres following quick change in load J Physiol (Lond) 184 511-534
- CLOSE R J 1972 Dynamic properties of mammalian skeletal muscles Physiol Rev 52 129-197
- COOKE P H & FAY F S 1972 Correlation between fiber length ultimate structure and the length-tension relationship of mammalian smooth muscle J Cell Biol 52 105-116
- CSAPO A 1962 Smooth muscle as a contractile unit Physiol Rev 42 suppl 5 7-33
- DOBRIN P B 1973 Influence of initial length on length-tension relationship of vascular smooth muscle Am J Physiol 226 664-670
- DRISKA S P & MURPHY R A 1978 Estimate of cellular force generated on an arterial smooth muscle with high actin-myosin ratio Blood Vessels 15 26-32
- DRISKA S P, DAMON D N & MURPHY R A 1978 Estimates of cellular mechanics in arterial smooth muscle Biophys J 24 525-540
- EDMAN K A P & NILSSON E 1972 Relationships between force and velocity of shortening in rabbit papillary muscle Acta physiol Scand 85 488-500
- FAY F S 1977 Isometric contractile properties of single isolated smooth muscle cells Nature 265 553-556



- FAY F & DELISE E 1973 Contraction of isolated smooth-muscle cells - structural changes Proc Natl Acad Sci 70: 641-645
- FAY F S COOKE P H & CANADAY M B 1976 Contractile properties of isolated smooth muscle cells In Physiology of smooth muscle (ed E Bülbring and M F Shuba) pp 249-264 Raven Press New York
- FORD L E HUXLEY A F & SIMMONS R H 1977 Tension responses to sudden length change in stimulated frog muscle fibres near slack length J Physiol (Lond) 269: 441-515
- GABELLA G 1975 Hypertrophy of intestinal smooth muscle Cell Tiss Res 163: 199-214
- GABELLA G 1976a Quantitative morphological study of smooth muscle cells of the guinea pig taenia coli Cell Tiss Res 170: 161-186
- GABELLA G 1976b Structural changes in smooth muscle cells during isotonic contraction Cell Tiss Res 170: 187-201
- GABELLA G 1976c The force generated by a visceral smooth muscle J Physiol (Lond) 263: 197-213
- GABELLA G 1977 Arrangement of smooth muscle cells and intramuscular septa in the taenia coli Cell Tiss Res 184: 195-212
- GABELLA G 1979a Hypertrophic smooth muscle I Size and shape of cells occurrence of mitoses Cell Tiss Res 201: 63-78
- GABELLA G 1979b Hypertrophic smooth muscle IV Myofilaments intermediate filaments and some mechanical properties Cell Tiss Res 201: 277-288
- GABELLA G 1979c Cell junctions and structural aspects of contraction Br med Bull 35: 213-218
- GORDON A M HUXLEY A F & JULIAN F J 1966 The variation in isometric tension with sarcomere length in vertebrate muscle fibres J Physiol (Lond) 184: 170-192
- GORDON A R & SIEGMAN H J 1971 Mechanical properties of smooth muscle I Length-tension and force-velocity relations Amer J Physiol 221: 1243-1249
- GÜLCH R W 1974 A critical analysis of myocardial force-velocity relations obtained from damped quick release experiments Basic Res Cardiol 69: 32-46
- HALPERN W SILVANY M J & WARSHAW D H 1978 Mechanical properties of smooth muscle cells in the walls of arterial resistance vessels J Physiol (Lond) 275: 85-101
- HAM A W 1969 Histology 6th ed Pitman med publ Co London
- HANRELL B B & ALPERT N R 1977 The mechanical characteristics of hypertrophied rabbit cardiac muscle in the absence of congestive heart failure Circulat Res 40: 0-25

- HAARDUNG V & LASZT L 1966 Die Beziehung zwischen Last und Verkürzungsgeschwindigkeit beim Gefäßmuskel *Angiologica* 3 100-113
- HELLSTRAND H 1977 Oxygen consumption and lactate production of the rat portal vein in relation to its contractile activity *Acta Physiol Scand* 100 91 106
- HELLSTRAND P 1979 Mechanical and metabolic properties related to contraction in smooth muscle *Acta Physiol Scand suppl* 464 pp 5-47
- HELLSTRAND H & JOHANSSON B 1975 The force-velocity relation in phasic contractions of venous smooth muscle *Acta Physiol Scand* 93 157 166
- HELLSTRAND P & JOHANSSON B 1979 Analysis of the length response to a force step in smooth muscle from rabbit urinary bladder *Acta Physiol Scand* 106 221 238
- HELLSTRAND H, JOHANSSON B & RINGBERG A 1972 Influence of extra cellular calcium on isometric force and velocity of shortening in depolarized venous smooth muscle *Acta Physiol Scand* 84 528 537
- HERLIHY J T & MURPHY R A 1974 Force-velocity and series elastic characteristics of smooth muscle from the hog carotid artery *Circulat Res* 34 461-466
- HILL A V 1938 The heat of shortening and the dynamic constants of shortening *Proc roy Soc B* 126 136-195
- HUXLEY A F & SIMMONS R M 1971 Proposed mechanism of force generation in striated muscle *Nature* 233 533 538
- HUXLEY H E 1971 The structural basis of muscular contraction *Proc roy Soc B* 178 131-149
- JEWELL B H & WILKIE D R 1958 An analysis of the mechanical components in frog's striated muscle *J Physiol (Lond)* 143 515-540
- JOHANSSON B 1973 Active state in the smooth muscle of the rat portal vein in relation to electrical activity and isometric force *Circulat Res* 32 246-258
- JOHANSSON B 1975 Structural and functional changes in rat portal veins after experimental hypertension *Acta Physiol Scand* 98 381 383
- JOHANSSON B 1978 Processes involved in vascular smooth muscle contraction and relaxation *Circulat Res* 43 Suppl 1 pp 14 20
- JONES A W, SOMLYO A P & SOMLYO A V 1973 Potassium accumulation in smooth muscle and associated ultrastructural changes *J Physiol (Lond)* 232 247 273
- JULIAN F J & MOSS R L 1976 The concept of a titin state in striated muscle *Circulat Res* 38 53 59
- KATZ B 1939 The relation between force and speed in muscular contraction *J Physiol (Lond)* 96 45 64

- LANNERGREN J 1978 The force-velocity relation of isolated twitch and slow muscle fibres of *Xenopus laevis* J Physiol (Lond) 283 501-521
- LARSEN J -J 1977 The ultrastructure of the pig and rabbit detrusor muscle fixed in vivo in the relaxed and contracted state bl Vet Med E Anat Hist Embryol 6 265-777
- MIASHIMA H & HANDA M 1969 The force-velocity relation and the dynamic constants of the guinea pig taenia coli J Physiol Soc Japan 31: 565-566
- MEISS R A 1978 Dynamic stiffness of rabbit mesotubarium smooth muscle effect of isometric length Amer J Physiol 234 C14-C26
- MERRILLEES M C R 1968 The nervous environment of individual smooth muscle cells of the guinea pig vas deferens J Cell Biol 37: 794-817
- MULVANY M J 1979 The undamped and damped series elastic components of a vascular smooth muscle Biophys J 26 401-414
- MULVANY M J & HALPERN W 1976 Mechanical properties of vascular muscle cells in situ Nature 260 617-619
- MULVANY M J & WARSHAW D M 1979 The active tension-length curve of vascular smooth muscle related to its cellular components J Gen Physiol 74 85-104
- MURPHY R A 1976 Contraction system function in mammalian smooth muscle Blood Vessels 13 1-23
- MURPHY R A, DRISKA S P & COHEN D M 1977 Variations in actin to myosin ratios and cellular force generation in vertebrate smooth muscles In Excitation-contraction coupling in smooth muscle (ed R Castells et al) pp 417-424 Elsevier/North Holland Amsterdam
- MURPHY R A, HERLIHY J T & MEGERIAN J 1974 Force-generating capacity and contractile protein content of arterial smooth muscle J Gen Physiol 64 691-705
- NAGASAWA J & SUZUKI T 1967 Electron microscopic study on the cellular interrelationships in the smooth muscle Tohoku J Exp Med 91 299-313
- PAGE S G & HUXLEY H E 1963 Filament lengths in striated muscle J Cell Biol 19 369-390
- PAUL R J, GLUCK E, RUEGG J C 1976 Cross bridge ATP utilization in arterial smooth muscle Pflügers Arch 361 297-299
- PEIPER U 1979 The rate of post contraction tension recovery in relation to type and period of activation in the isolated rat portal vein Pflügers Arch 382 R25
- PETERSON C M, GOSS R J & ATRYZEK W 1974 Hypertrophy of the rat urinary bladder following reduction of its functional volume J Exp Zool 187 121-126
- PETERSON J W & PAUL R J 1974 Effects of initial length and active shortening on the smooth muscle contractility Amer J Physiol 227 1019-1024

ROEIS H 1968 Mikroskopische Technik Oldenburg München

ROSEBLUTH J 1965 Smooth muscle An ultrastructural basis for the dynamics of its contraction Science 148 1 37-1339

RÜDEL R & TAYLOR S R 1971 Striated muscle fibers: Inactivation of contraction induced by shortening Science 172 387-388

RIEGG J C 1971 Smooth muscle tone Physiol Rev 51 201-248

SIMMONS R M & JEWELL B R 1974 Mechanics and models of muscular contraction In Recent advances in physiology n 9 (ed R J Linden) pp 87-147 Churchill Livingstone Edinburgh and London

SJOLIN L, HELLSTRAND P & CLEMENTZ B 1978 An apparatus for mechanical experiments on isolated smooth muscle Acta Physiol Scand 102: 32A-33A

SORILYO A P & SORILYO A V 1975 Ultrastructure of smooth muscle In Methods in pharmacology (ed E E Daniel and D M PATON) pp 3-46 Plenum Press New York

SPEIDEN R W 1960 The effect of initial strip length on the noradrenaline induced isometric contraction of arterial strips J Physiol (Lond) 154 15-25

STEPHENS M L & KROMER U 1971 Series elastic component of tracheal smooth muscle Amer J Physiol 220 1890-1895

STEPHENS M L & VAN NIEKERK W 1977 Isometric and isotonic contractions in airway smooth muscle Can J Physiol Pharmacol 55: 833-838

STEPHENS M L, KROEGER E & MEHTA J A 1969 Force-velocity characteristics of respiratory airway smooth muscle J Appl Physiol 26 685-692







ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 484

# UTERINE SECRETOMOTOR INNERVATION

By

MARGARETA HAMMARSTRÖM

STOCKHOLM 1980





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 484

From the Department of Physiology I, Karolinska Institutet,  
104 01 Stockholm, Sweden

# UTERINE SECRETOMOTOR INNERVATION

By

MARGARETA HAMMARSTRÖM

Apart from some previously unpublished results, the present thesis is based on the following papers, which are being referred to by their roman numerals.

- I Hammarström, M & Sjöstrand, N O 1979 Evidence for a cholinergic secretory innervation of the guinea pig endometrium. *Acta Physiol Scand* 106 11—15
- II Hammarström, M & Sjöstrand N O 1980 Acetylcholinesterase positive nerves in the guinea-pig endometrium *Acta Physiol Scand* (In Press)
- III Hammarström, M 1980 Endometrial cholinergic secretory responses during estrous cycle, pregnancy and after estrogen and/or progesterone treatment of the guinea pig. *Acta Physiol Scand* (In Press)

# CONTENTS

I.	INTRODUCTION	4
1	Historical survey	4
2	Anatomical survey	5
3	The reproductive cycle	6
II.	MATERIAL AND METHODS	8
III.	RESULTS, COMMENTS, AND CONCLUSIONS	11
1	Secretory nerves to the guinea pig endometrium	11
a.	Presence and nature of endometrial secretomotor innervation	
b.	Possible origin and course of the cholinergic secretomotor nerves	
2	Secretory responses to autonomic drugs	12
3	Alterations in secretory responses after female sex hormone treatment, in estrous cycle and during pregnancy	12
4	Resting secretion level and endometrial carbohydrate concentration	15
IV.	GENERAL DISCUSSION	16
V.	SUMMARY	18
VI.	ACKNOWLEDGEMENTS	19
VII.	REFERENCES	20

# I. INTRODUCTION

## 1. Historical survey

Uterine Innervation has been the subject of many comprehensive studies and surveys. Detailed accounts of the early literature are given by e.g. Lee (1841 1858) Frankenhäuser (1867), Labhardt (1906) Hellerman (1927) and Krantz (1959) The reviews of Labhardt (1906), Dahl (1916, 1924) Mabuchi (1924) Hellerman (1927) Davis (1933) and Gruber (1933) present thorough surveys of the work performed on the uterine innervation at the turn of the century Good accounts of literature on research on uterine innervation during recent decades are to be found in the texts by e.g. Reynolds (1949 1965) Krantz (1959) Marshall (1970) Bell (1972) Owman, Sjöberg & Sjöstrand (1974) Thorbert (1978)

However most reviews on uterine innervation reveal that the main interest has been focused on efferent myometrial or vascular innervation or sensory innervation. The possible efferent innervation of the endometrium has attracted considerable less morphological interest and from a physiological point of view virtually no interest. The existence of nerves to the human mucosa of the have even been denied by e.g. Mabuchi (1924) and Schröder (1930)

Yet endometrial nerves have been described in many histological studies. Those first demonstrating the penetration of nerves to the endometrium of mammals using the light microscope appear to be the studies of Kilian (1841) and Frankenhäuser (1867), the latter solely describing nerves to the cervical part of the mucosa.

With the development of metal impregnation techniques and the introduction of supra- and intravital methylene blue staining further and more detailed studies appeared Nerves to the epithelial and glandular layers of the human endometrium were described by Patenko (1880) and Gawronsky (1894). Këullin (1894) also using silver impregnation confirmed the existence of such nerves in the endometrium of several species including the guinea pig. Gentes (1902) using intravital methylene blue staining was able to demonstrate nerves to the epithelial and glandular linings of the endometrium of the rabbit and rat and Lab-

hardt (1906) using both intravital methylene blue staining and the Golgi method demonstrated nerves to the submucosa of the rabbit and human endometrium. Supravital methylene blue staining was used by Hoogkamer (1913) who in various species e.g. rabbit, cat, cattle and human was able to demonstrate nerves to the epithelium. Using the same method, Dahl (1916, 1924) observed nerves to the mucosa of the human uterus although he did not pay any greater attention to them. Mabuchi (1924) and Davis (1933) using silver impregnation techniques and intravital methylene blue staining respectively could only demonstrate nerves to the cervical part of the human mucosa. Nerves to the endometrium of the cat were demonstrated by Feldmann (1935) He also noted that part of the endometrial nerves degenerated after hypogastric nerve section.

Several later authors using various metal impregnation techniques have demonstrated nerves to the human endometrium. Thus nerves to the basal third of the endometrium of the adult uterus were observed by State & Hirsch (1941) Freeman (1946) Koppen (1950) and Krantz (1959). Jacobson (1953) noted nerves to the basal two thirds while nerves to the glandular and epithelial linings of the human adult uterus were seen by e.g. Nishimura (1954) and Moricard & Giro (1958) Similar findings were made on the fetal human uterus (Casaglia & Giro 1963) and on the newborn human uterus (Pribor 1951) Nerves to the submucosa of the corpus and a subepithelial plexus in the cervix of the fetal human uterus were also reported by Brown & Hirsch (1941) using the Masson trichrome stain.

Also in several other species nerves to the glandular and epithelial linings of the endometrium have been demonstrated during the last decades as e.g. in the rabbit (Coujard 1951 Moricard & Giro 1958) and in the rat (Moricard & Giro 1958) as well as in the rhesus macaque (Jacobson & Nieves 1961 Laxman 1965) Nerves to the guinea pig endometrium were denied by Moricard & Giro (1958) while Jacobson & Nieves (1961) demonstrated such nerves with intravital methylene blue staining, thus confirming the statements of Këullin (1894)

Recent advance in histochemistry has provided more selective nerve staining techniques as e.g. the Falck-Hillarp method for adrenergic nerves and the Koelle-Holmstedt method for acetylcholinesterase positive nerves. Thus, Coupland (1962, 1969) demonstrated a rich supply of acetylcholinesterase positive nerves to the human cervical mucosa and a similar but sparser supply to the mucosa of the corpus. An acetylcholinesterase positive plexus spreading all over the rat endometrium was studied in detail by Adham & Schenk (1969). This plexus was accompanied by a scanty adrenergic plexus situated in the basal endometrium. Adrenergic nerves to the basal part of the endometrium were also observed in the rabbit (Owman & Sjoberg 1966), in the rat (Adham & Schenk 1969), in the human (Dallenbach & Vonderlin 1973), and in the guinea-pig (Thorbert et al. 1977).

Using electron microscopy Kihlhel & Beier (1976) recently described nerve terminals containing synaptic vesicles to the glandular cells of the endometrium of several mammalian species. Regions of close apposition between nerve terminals, partly losing their Schwann sheath, and glandular cells were observed. In many cases the basal lamina was lacking and the cleft was only about 20 nm suggesting an intimate neuroeffector arrangement.

As evident from this survey nerves to the secretory linings have been repeatedly observed during more than a century. Besides sensory function, a secretomotor function of these nerves also has been postulated by several authors e.g. Schofield (1952), Marshall (1970), Dallenbach & Vonderlin (1973) and Kihlhel & Beier (1976). However no study of a possible secretomotor function of these nerves seems to have been made. The absence of such studies is probably due to the difficulty in estimating changes in uterine mucus caused by e.g. nerve stimulation since the amount secreted is very small, viscous and hard to collect. Furthermore in *in vivo* experiments, there is also a problem in distinguishing active secretion of a small amount of sticky uterine fluid from passive squeezing out of the fluid due to myometrial contraction when uterine nerves are stimulated. In addition to this a secretory response to nerve stimulation might be jeopardized by a concomitant circulatory effect because vasoconstrictor and dilator fibres run among other uterine nerves.

In order to minimize these problems and to standardize the conditions the present study was performed on isolated everted uterine horns. Carbohydrate was used as marker of secretion. The guinea-pig was chosen as experimental animal for the following reasons. a) the anatomy of the uterus and the nerves is clear and easy accessible for experimental conditions. b) It has a reproductive cycle reminding of the human (Thorburn, Challis & Robinson 1977). c) Many basic facts of the uterine innervation and its organization are well settled.

## 2. Anatomical survey (Fig. 1)

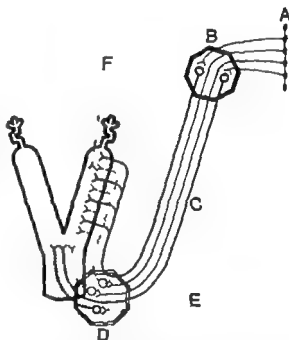


Fig. 1 Schematic drawing of the guinea-pig uterus and its innervation. A. Thoraco-lumbar sympathetic chain. B. Inferior mesenteric ganglion. C. Hypogastric nerve. Part of the fibres in the hypogastric nerve are preganglionic and part of them are postganglionic, the latter relaying in the inferior mesenteric ganglion. D. Paracervical ganglion through which most of the nerves to the uterus pass. Part of the sympathetic supply to the uterus relay in this ganglion. E. A possible uterine innervation via the pelvic nerve has been debated. If present, the fibres are supposed to relay in the paracervical ganglion (D). F. Nerves via the conoarterial connection to the uterus: adrenergic nerves are supposed to supply the myometrium of the subal part of the uterine horn. (Modified 1973 and Thorbert 1978)

The guinea pig has a bicornuate uterus. The uterine nervous supply is derived via two main pathways: a) the paracervical ganglia and b) the costouterine connexion (the suspensory ligament) (cf. Gabella 1976, Thorbert et al. 1977).

a) The two paracervical ganglia receive nerves from each hypogastric nerve and also from each pelvic nerve (see below). The hypogastric nerve descends from the inferior mesenteric ganglion which receives fibres from the thoraco-lumbar sympathetic chain. According to Thorbert (1978) one half of the adrenergic innervation reaching the uterus via the paracervical ganglia consists of short postganglionic neurons.

The pelvic nerve supply to the uterus via the paracervical ganglia of various mammals has been debated. With respect to the myomotor supply some authors have stated that there is no such supply at all (Langley & Anderson 1896a, vide e.g. Sjöberg 1967, Theobald 1973). Schofield (1952) noted an excitatory effect on the rabbit myometrium in one fourth of experiments on stimulating the pelvic nerve. Other statements are that there is an excitatory myomotor supply (Körner 1865, Basch & Hofmann 1877, Fellner 1887, Shabanah, Toth & Maughan 1964, Carsten 1968) or an inhibitory myomotor supply (Dahl 1916, 1924, Basch & Hofmann (1877) and Bell (1972) suggested a cholinergic vasodilator innervation via pelvic nerve of the guinea-pig, however this has not been proved.

b) The adrenergic uterine nervous supply via the costouterine connexion mainly reaches the tubal part of the horn of the guinea pig (Thorbert et al. 1977). These nerves consist of long postganglionic fibres and might be derived from the aortico-renal plexus (Kuntz 1947, Thorbert et al. 1977).

### 3. The reproductive cycle (Fig. 2 & 3)

The adult female guinea pig has an estrous cycle of 16 days duration. The cycle can be roughly divided into *proestrus* lasting for 1–1.5 days, *estrus* often nocturnal, lasting for 7–24 hours, *metestrus* with a duration of —4 days followed by *diestrus* (cf. Fig. 2) (Stockard & Papanicolaou 1917, 1919, Ishii 1928, Young 1936/37, Burgos & Wislocki 1956, 1958).

A rough estimate of the occurrence of the estrus phase can be made by inspection. Thus the vaginal

closure membrane is open for 2–4 days during estrus. From the appearance of vaginal smears the estrus phase could be further divided. This renders the possibility to better determine the time of ovulation (vide Stockard & Papanicolaou 1917, 1919, Sells 1922, Young 1936/37, Bland & Donovan 1970). The day of ovulation is designed day 1 of the cycle.

If conception occurs, the ovum reaches the uterus at the end of day 3 or the beginning of day 4 (Bischoff 1852, Squier 1932). Implantation occurs on day 6–7 (Bischoff 1852, von Spee 1901, Emrys-Roberts 1909/10, Sansom & Hill 1931 and Blandau 1949). The gestational time is 63–68 days (Bischoff 1852, Phoenix 1970). 1–4 hours post partum the para gets into estrus (Bischoff 1852, Ishii 1928, Bolling et al. 1939, Phoenix 1970). She ovulates and conceives if fertilized.

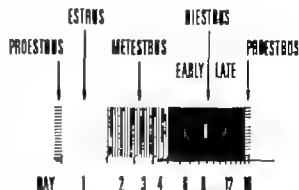


Fig. 2 Schematic illustration showing the estrous cycle of the guinea pig. Ovulation occurs at day one. (Days on logarithmic scale.)

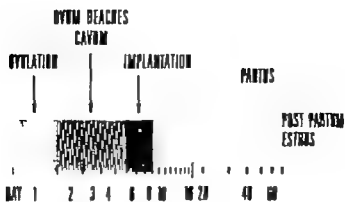


Fig. 3 Schematic illustration of the sequences of pregnancy of the guinea-pig. (Days on logarithmic scale.)



## II MATERIAL AND METHODS

**Animals.** Sexually mature mottled or albino virgin guinea pigs (300—500 g) were used.

**Hormonal treatment** In paper I estradiol benzoate 6 mg/kg in combination with hydroxyprogesterone caproate 150 mg/kg (Primobolston®) was injected every second day for 10—14 days. In paper III estradiol valerate 6 mg/kg (Progynon®) and hydroxyprogesterone caproate 150 mg/kg (Proluton®) alone or in combination was given every second day for 10—14 days. In paper II estradiol benzoate 6 mg/kg in combination with hydroxyprogesterone caproate 150 mg/kg (Primobolston®) were injected every third day for 6—8 days.

**Estrous cycle determination (III)** The animals were controlled daily concerning the status of the vaginal closure membrane. Only animals showing regular cycles were chosen. After at least two normal cycles, vaginal smears were taken when the membrane was open. The day of maximum cornification before leucocytic influx was designated day I (cf. Stockard & Papanicolaou 1917, 1919; Bland & Donovan 1970; Thorbert 1978).

**Determination of pregnancy (III)** 4—5 females were caged with one male. The females were controlled daily concerning the status of the vaginal closure membrane. Pregnancy was estimated from the second day of opening and controlled by palpation early in pregnancy.

**Anaesthesia.** All operations were performed under sodium pentobarbital anaesthesia when necessary supplemented with ether (I & III).

**Operative procedures.** Bilateral denervations were undertaken 10—14 days before an experiment was performed. (i) The *hypogastric nerve* was sectioned 1 cm below the inferior mesenteric ganglion and about 2 mm of the nerve was removed. Each *pelvic nerve* was similarly sectioned 1 cm proximal to its crossing of the iliac artery. The *paracervical ganglia* were destroyed with phenol (8.5 M) and the nerves of the *costouterine connexion* were destroyed by a tight ligature dipped in 8.5 M phenol.

**Bilateral oophorectomy (III)** was performed via dorsal incisions 3 weeks before hormone treatment was started.

**Preparation (dissecting and mounting pro-**

**cedures)** (cf. paper I) All animals were stunned and bled, the uterus and parametrial tissue were dissected out. The uterus was divided through the cervix, each horn everted and mounted on a platinum electrode. Each horn was put in an organ bath of 25 ml or 100 ml (late pregnancy horns) containing Tyrode solution (Fig. 4).

Many factors contributed to the fact that the whole uterus was used. The innervation is richest

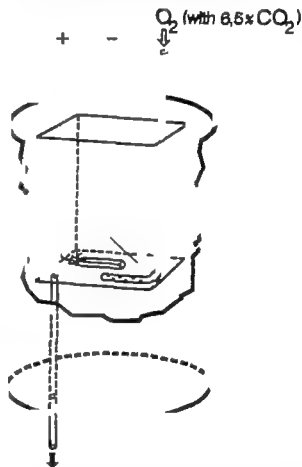


Fig. 4 Schematic drawing of experimental set-up. Tyrode solution (36 °C) is poured into the organ bath from above. White arrow shows tube for carbogen gas supply. The everted uterine horn is mounted on an platinum electrode, shielded except for the part situated within the uterine horn. The electrode is supplied with a small glass knob in order to avoid penetration of the uterine wall. The other electrode is isolated except for its last part. Black arrow indicates the outlet for Tyrode solution.

in the cervical part of the mucosa of human and rat (Coupland 1962, 1969 Adham & Schenk 1969). The cervix alone is however impossible to evert and it is difficult to identify by eye the limit of the cervical part of the mucosa which might render an insecurity concerning preparation from experiment to experiment.

**Experimental performance** Experiments were performed following the schedule given in paper I.

After 80 min accommodation in ordinary Tyrode solution changed every 20 min, the sampling was started. Five 10 min sampling periods were performed in Tyrode solution where glucose was replaced by NaCl. The sampling periods were separated by resting periods of 20 min in ordinary Tyrode solution. The first, third and fifth sampling periods were used for determination of carbohydrate resting secretion level. The second and fourth periods were used for studying the effect of nerve field stimulation or administration of drugs, or both. In certain experiments only three sampling periods were performed. In these cases there were two sampling periods to determine resting secretion level surrounding one stimulation period.

**Estimation of secretion. Choice of marker and method** As the endometrium undergoes cyclical changes a marker not affected by such changes is hardly possible to find. Since the uterine mucus is rich in carbohydrates of many different types, it was necessary to use a method for determination of total carbohydrate amounts. The method chosen was the indole-sulfuric acid method for total carbohydrates (Dishe & Popper 1926) used for determination of total carbohydrate amount in cervical mucus by Shettles (1951) and Shettles, Dishe & Osnes (1951).

**Analysis of samples.** The volumes from the sampling periods were lyophilized (I III) or evaporated (at 40 °C late pregnancy experiments, III). The dry residue from the sampling periods was dissolved in 0.5 ml (I ml) water and 4.5 ml (9 ml) 14.4 M  $H_2SO_4$ . The samples were centrifuged and the supernatant (3 ml or 4.5 ml) was collected. To it was added 0.10 ml (0.15 ml)  $8.5 \times 10^{-2}$  M indole (figures between brackets for samples from experiments late in pregnancy). The sample was then heated for 10 min in 100 °C. The carbohydrate concentration was determined spectrophotometrically (470 nm) using an internal standard.

#### *Determination of mucosal carbohydrate content*

After the experiment the endometrial layer was separated from the myometrium and a small piece was taken for analysis. Each piece was homogenized by grinding with quartz sand in a mortar containing 0.5 ml of 14.4 M  $H_2SO_4$ . Rhinal was performed with 4 ml 14.4 M  $H_2SO_4$  and 0.5 ml distilled water. After shaking the homogenate was centrifuged. 4.5 ml of the supernatant was removed and to it 0.15 ml  $8.5 \times 10^{-2}$  M indole was added and the sample analysed as described above.

**Stimulations** Field stimulation of nerves was applied at a standard rate of 0.5 Hz, 0.5 ms, 30 V. Nerve stimulation was applied with a frequency of 5 Hz (0.1 ms, 30 V) for 10 s every 30 s. Carbachol was administered in doses of  $5.5 \times 10^{-10}$ – $5.5 \times 10^{-9}$  M.

**Calculation and statistics** Because of the rather great variance between different experiments in resting level of carbohydrate secretion all results were calculated as a percent of resting secretion level using the mean of the three (two) resting periods as matching value. Each type of experiment was performed on uteri from at least three animals.

The Student's t-test or when necessary the Welch approximate t test was used for statistical comparison of experiments (Remington & Schork 1970).

**Drugs** Apart from the hormones obtained from Schering AG drugs used were: carbamylcholine chloride (Carbachol) (Sigma) scopolamine hydrobromide (Sigma) phenolamine chloride (Ciba), sotalol hydrochloride (MJ 1999 Regis Chemical Company) isoprenaline sulphate (Abbott), methoxamine hydrochloride (BDH), tetrodotoxin (TTX) (Sigma).

**Tyrode solution composition** NaCl  $137 \times 10^{-3}$  KCl  $2.7 \times 10^{-3}$  MgCl  $4.9 \times 10^{-3}$   $CaCl_2$   $1.8 \times 10^{-3}$   $NaHCO_3$   $1.2 \times 10^{-3}$   $Na_2HPO_4 \cdot 2H_2O$   $3.1 \times 10^{-4}$  M  $11 \times 10^{-2}$  mol of glucose was added/l of ordinary Tyrode solution and  $3.6 \times 10^{-2}$  mol of NaCl to each liter of glucose free Tyrode solution.

**Demonstration of acetylcholinesterase positive nerves (II)** The modified Koelle Holmstedt method of Karnovsky & Roots (1964) was used for identifying AChE positive nerves. The uterus was carefully dissected out and cut into 5–8 mm pieces. The pieces were either directly frozen (–30 °C) or frozen after 1–16 h of fixation in ice-cold 10% formalin containing 1

Uvnäs 1960 Sjöqvist 1963 Folkow & Neil 1971) A secretory innervation of vaginal glands via the hypogastric nerve has been suggested by Davis (1934) Furthermore, the secretory innervation of the dog prostate is cholinergic and sympathetic. Most fibres seem to be preganglionic and run in the hypogastric nerve (vide Sjöstrand 1976) Also cholinergic vasodilator fibres to the penis of rabbit seem to have a preganglionic supply via the hypogastric nerve (Sjöstrand & Klinge 1979)

Secretomotor innervation via the pelvic nerve and/or the costouterine connexion cannot be excluded. However possible secretomotor fibres passing these routes can hardly contribute to a very prominent proportion of the secretomotor innervation. Here it is to be mentioned, that according to Thorbert et al. (1977) no acetylcholinesterase positive nerves are present in the costouterine connexion. A possible uterine innervation derived from the pelvic nerve is subject to different opinions (see Introduction)

The above statements concerning pathways and synaptic relays of the secretomotor fibres are guarded and not too precise. This must be so because there is not only the roughness of the method to be considered. Also possible phenomena as denervation supersensitivity of effector cells deprived of their original innervation but excited by minute amounts of diffused transmitter and reinnervation of effector cells from sprouting terminals deriving from unsevered nerves have to be taken into account

## 2. Secretory responses to autonomic drugs (I)

The results documented above led to the conclusion that uterine secretory responses were evoked by stimulation of cholinergic nerves having their main course—pre- or postganglionic—in the hypogastric nerve. The receptors on the secretory cells were apparently muscarinic

Accordingly it was considered to be of interest to study the effect of a muscarinic drug. Carbachol was chosen because of its high potency and resistance to acetylcholinesterase. Furthermore, in the extended study (see below) transmural stimulation of nerves was impossible to accomplish in pregnancy (day 16–42) which made experiments with a muscarinic drug necessary

Carbachol ( $5.5 \times 10^{-6}$  M) increased secretion to the same extent as did transmural stimulation, the

response was dose dependent with a sharp threshold at  $5.5 \times 10^{-9}$  M whereafter maximum rapidly occurred. Scopolamine ( $1.3 \times 10^{-6}$  M) abolished the effect of carbachol. This further substantiated the muscarinic nature of the excitatory receptors of the secretory cells and is in line with the studies of Marco (1930) Shih, Kennedy & Huggins (1940) and Kaulla et al (1957) which demonstrated alterations in uterine secretion after pilocarpine administration to bitch or woman.

Neither the  $\alpha$ -adrenoceptor stimulant methoxamine ( $4 \times 10^{-6}$  M  $4 \times 10^{-6}$  M) nor the  $\beta$ -adrenoceptor agonist isoprenaline ( $2 \times 10^{-6}$  M) produced any clearcut effects on endometrial secretion. Consequently no evidence of any significant adrenergic mechanism affecting uterine secretion was obtained in these experiments.

## 3. Alterations in secretory responses after female sex hormone treatment, in estrous cycle and during pregnancy (I III)

The experiments described above elucidating endometrial innervation and its sources were performed on animals purposely brought into artificial secretory phase. For natural reasons it appeared to be of interest to investigate possible secretory responses during normal reproductive cycle of the guinea pig and after excess of estrogen and/or progesterone treatment.

*Combined treatment with estrogen and progesterone* a) Intact animals (I) treated with the commercial product Primoluton®. Field stimulation of nerves produced a secretory response of +71 %. Carbachol ( $5.5 \times 10^{-6}$  M) raised the secretion to the same level (+68 %). Threshold concentration was  $5.5 \times 10^{-9}$  M (see above)

b) Spayed animals (III) treated with the commercial products Progynon® (estrogen) and Pro-luton® (progesterone). Transmural stimulation of nerves increased secretion by 36 %. Carbachol produced an increase in secretion by about the same magnitude (+41 % at  $5.5 \times 10^{-6}$  M and +49 % at  $5.5 \times 10^{-7}$  M) as did transmural stimulation of nerves. The sensitivity to carbachol was similar to that of the intact animals treated with both hormones. (The differences between these two groups in degree of response to transmural stimulation or to carbachol are not statistically significant.)

in the cervical part of the mucosa of human and rat (Coupland 1962, 1969; Adham & Schenk 1969). The cervix alone is however impossible to evert and it is difficult to identify by eye the limit of the cervical part of the mucosa which might render an insecurity concerning preparation from experiment to experiment.

**Experimental performance** Experiments were performed following the schedule given in paper I.

After 80 min accommodation in ordinary Tyrode solution changed every 20 min, the sampling was started. Five 10 min sampling periods were performed in Tyrode solution where glucose was replaced by NaCl. The sampling periods were separated by resting periods of 20 min in ordinary Tyrode solution. The first, third and fifth sampling periods were used for determination of carbohydrate resting secretion level. The second and fourth periods were used for studying the effect of nerve field stimulation or administration of drugs, or both. In certain experiments only three sampling periods were performed. In these cases there were two sampling periods to determine resting secretion level surrounding one stimulation period.

**Estimation of secretion. Choice of marker and method** As the endometrium undergoes cyclical changes a marker not affected by such changes is hardly possible to find. Since the uterine mucus is rich in carbohydrates of many different types, it was necessary to use a method for determination of total carbohydrate amounts. The method chosen was the indole-sulfuric acid method for total carbohydrates (Dishe & Popper 1926) used for determination of total carbohydrate amount in cervical mucus by Shetles (1951) and Shetles, Dishe & Osmos (1951).

**Analysis of samples.** The volumes from the sampling periods were lyophilized (I, III) or evaporated (at 40 °C) (late pregnancy experiments, III). The dry residue from the sampling periods was dissolved in 0.5 ml (1 ml) water and 4.5 ml (9 ml) 14.4 M  $H_2SO_4$ . The samples were centrifuged and the supernatant (3 ml or 4.5 ml) was collected. To it was added 0.10 ml (0.15 ml)  $8.5 \times 10^{-3}$  M indole (figures between brackets for samples from experiments late in pregnancy). The sample was then heated for 10 min in 100 °C. The carbohydrate concentration was determined spectrophotometrically (470 nm) using an internal standard

**Determination of mucosal carbohydrate content** After the experiment the endometrial layer was separated from the myometrium and a small piece was taken for analysis. Each piece was homogenized by grinding with quartz sand in a mortar containing 0.5 ml of 14.4 M  $H_2SO_4$ . Rinsing was performed with 4 ml 14.4 M  $H_2SO_4$  and 0.5 ml distilled water. After shaking the homogenate was centrifuged. 4.5 ml of the supernatant was removed and to it 0.15 ml  $8.5 \times 10^{-3}$  M indole was added and the sample analysed as described above.

**Stimulations.** Field stimulation of nerves was applied at a standard rate of 0.5 Hz, 0.5 ms, 30 V. Nerve stimulation was applied with a frequency of 5 Hz (0.1 ms, 30 V) for 10 s every 30 s. Carbachol was administered in doses of  $5.5 \times 10^{-10}$ – $5.5 \times 10^{-8}$  M.

**Calculation and statistics** Because of the rather great variance between different experiments in resting level of carbohydrate secretion all results were calculated as a percent of resting secretion level using the mean of the three (two) resting periods as matching value. Each type of experiment was performed on uteri from at least three animals.

The Student's t-test or when necessary the Welch approximate t-test was used for statistical comparison of experiments (Remington & Schork 1970).

**Drugs** Apart from the hormones obtained from Schering AG drugs used were, carbamylcholine chloride (Carbachol) (Sigma), scopopolamine hydrobromide (Sigma), phenolamine chloride (Ciba), sotalol hydrochloride (MJ 1999 Regis Chemical Company), isoprenaline sulphate (Abbot), methoxamine hydrochloride (BDH), tetrodotoxin (TTX) (Sigma).

**Tyrode solution composition** NaCl  $13.7 \times 10^{-2}$  M, KCl  $2.7 \times 10^{-2}$  M,  $MgCl_2$   $4.9 \times 10^{-3}$  M,  $CaCl_2$   $1.8 \times 10^{-3}$  M,  $NaHCO_3$   $1.2 \times 10^{-2}$  M,  $Na_2HPO_4 \cdot 2H_2O$   $3.1 \times 10^{-4}$  M,  $1.1 \times 10^{-2}$  mol of glucose was added/l of ordinary Tyrode solution and  $5.6 \times 10^{-4}$  mol of NaCl to each liter of glucose free Tyrode solution.

**Demonstration of acetylcholinesterase positive nerves (II).** The modified Koelle Holmstedt method of Karnovsky & Roots (1964) was used for identifying AChE positive nerves. The uterus was carefully dissected out and cut into 5–8 mm pieces. The pieces were either directly fixed (–30 °C) or frozen after 1–16 h of ice-cold 10 % formalin.

(Karnovsky & Roots) followed by 1—4 days in ice-cold "gum-sucrose" solution (0.88 M sucrose containing 1% gum arabic) (Holt, Hobbiger & Pawan 1960). Some unfixed sections were post fixed in formalin according to El-Badawi & Schenk (1967). Sections (10  $\mu$ ) were cut in a

cryostat. Time in the incubation mediums recommended by Karnovsky & Roots was 1—5 h. Iso-OMPA ( $4 \times 10^{-4}$  M) was used to inhibit nonspecific cholinesterase activity. For general staining of the sections eosin or lichtgrün was used.

### III. RESULTS COMMENTS AND CONCLUSIONS

#### 1. Secretory nerves to the guinea-pig endometrium (I & II)

The aim of these studies was to collect evidence of a secretomotor function of certain endometrial nerves. Unspayed guinea-pigs, whose endometria had been brought into artificial secretory phase were used (see Material and Methods)

##### a. Presence and nature of endometrial secretomotor innervation

At the standard field stimulation rate an increase in secretion of 70 % was seen. This response was abolished by TTX ( $3 \times 10^{-7} M$ ). *Scopolamine* ( $1.3 \times 10^{-6} M$ ) levelled away the secretory response while *sotalol* ( $1.8 \times 10^{-7} M$ ) was without overt effect. After *phenolamine* ( $1.6 \times 10^{-6} M$ ) the secretory response reached +84 % (This value was not statistically significantly higher than the value obtained after field stimulation solely)

The susceptibility of the field stimulation response to TTX, suggested a neurogenic origin. Since the response was abolished by *scopolamine* the nerves were likely to be cholinergic and the receptors on the endometrial secretory cells muscarinic. These suggestions were further supported by the demonstration of acetylcholinesterase positive nerves in the guinea-pig endometrium (II). Such nerves have been reported in the human endometrium (Coupland 1962, 1969) and Adham & Schenk (1969) have demonstrated a mucosal acetylcholinesterase positive plexus to the epithelial and glandular layers of the rat endometrium. However some authors have not been able to demonstrate such nerves, in human, rabbit or guinea-pig endometrium (Owman & Sjöberg 1966, Jordan 1970 Thorbert et al. 1977). The diversity in opinion is probably due to difficulties involved in displaying endometrial nerves since it is difficult to obtain a proper fixation attended by a desired staining (cf Thorbeck 1973, II).

From the results it can be concluded that the guinea-pig endometrium possesses a cholinergic secretory innervation. Presumably this conclusion could be extended to other mammals, at least to man and rat.

##### b. Possible origin and course of the cholinergic secretomotor nerves

*Stimulation of extrinsic nerves.* Stimulation of the hypogastric nerve produced an increase in secretion by about 40 % while pelvic nerve stimulation was without overt effect.

*Experiments on denervated uteri.* Field stimulation after section and degeneration of the hypogastric nerve resulted in a secretory response of +26 % i.e. about one-third of the control level. Destruction of *paracervical ganglia* produced a further decrease in the obtained secretory responses to one-sixth (+11 %). Chronic interruption of the pelvic nerve as well as of the *cervico-uterine connexion* produced no definite reduction in uterine secretory responses (+56 % and +52 % respectively)

Consequently the hypogastric nerve seems to be the main secretomotor pathway. Part of the fibres are postganglionic, part of them are preganglionic, the latter relaying in the paracervical ganglia. A similar pattern with pre and postganglionic fibres in the hypogastric nerve has been suggested for uterine myomotor nerves in the cat and rabbit (Langley & Anderson 1896b) in the rabbit (Varagic 1956) and in the guinea-pig (Rüsse & Marshall 1970). Feldmann (1935) showed degeneration of part of the uterine nerves of the cat following section of the hypogastric nerve. Furthermore, this organization is in agreement with studies on distribution of adrenergic terminals and the presence of the adrenergic transmitter noradrenaline, in the myometrium (for ref see Sjöberg 1967 Owman et al. 1974 Thorbert 1978). These studies show that postganglionic adrenergic fibres pass via the paracervical ganglia and that preganglionic fibres relay there, i.e. on short adrenergic neurones. Also cholinergic vasodilator fibres relay in the paracervical ganglia, their preganglionic source being more obscure (Bell 1972, 1974a, b).

From an organizational point of view the uterine secretomotor fibres would be regarded as belonging to the sympathetic system. Cholinergic sympathetic innervation to sweat glands and blood vessels of skeletal muscle of certain species is well documented (vide Langley 1891 1895).

Uvnäs 1960 Sjöqvist 1963 Folkow & Nell 1971) A secretory innervation of vaginal glands via the hypogastric nerve has been suggested by Davis (1934) Furthermore, the secretory innervation of the dog prostate is cholinergic and sympathetic. Most fibres seem to be preganglionic and run in the hypogastric nerve (vide Sjöstrand 1976) Also cholinergic vasodilator fibres to the penis of rabbit seem to have a preganglionic supply via the hypogastric nerve (Sjöstrand & Klinge 1979)

Secretomotor innervation via the pelvic nerve and/or the costouterine connexion cannot be excluded. However possible secretomotor fibres passing these routes can hardly contribute to a very prominent proportion of the secretomotor innervation. Here it is to be mentioned, that according to Thorbert et al (1977) no acetylcholinesterase positive nerves are present in the costouterine connexion. A possible uterine innervation derived from the pelvic nerve is subject to different opinions (see Introduction)

The above statements concerning pathways and synaptic relays of the secretomotor fibres are guarded and not too precise. This must be so because there is not only the roughness of the method to be considered. Also possible phenomena as denervation supersensitivity of effector cells deprived of their original innervation but excited by minute amounts of diffused transmitter and innervation of effector cells from sprouting axons deriving from unsevered nerves have to be taken into account.

## 2. Secretory responses to autonomic drugs (I)

The results documented above led to the conclusion that uterine secretory responses were evoked by stimulation of cholinergic nerves having their main course—pre- or postganglionic—in the hypogastric nerve. The receptors on the secretory cells were apparently muscarinic.

Accordingly it was considered to be of interest to study the effect of a muscarinic drug. Carbachol was chosen because of its high potency and resistance to acetylcholinesterase. Furthermore, in the extended study (see below) transmural stimulation of nerves was impossible to accomplish in pregnancy (day 16—42), which made experiments with a muscarinic drug necessary.

Carbachol ( $5.5 \times 10^{-5}$  M) increased secretion to the same extent as did transmural stimulation, the

response was dose dependent with a sharp threshold at  $5.5 \times 10^{-6}$  M whereafter maximum rapidly occurred. Scopolamine ( $1.3 \times 10^{-6}$  M) abolished the effect of carbachol. This further substantiated the muscarinic nature of the excitatory receptors of the secretory cells and is in line with the studies of Marco (1930) Shih, Kennedy & Huggins (1940) and Kaulla et al (1957) which demonstrated alterations in uterine secretion after pilocarpin administration to bitch or woman.

Neither the  $\alpha$ -adrenoceptor stimulant methoxamine ( $4 \times 10^{-6}$  M  $4 \times 10^{-4}$  M) nor the  $\beta$ -adrenoceptor agonist isoprenaline ( $2 \times 10^{-6}$  M) produced any clearcut effects on endometrial secretion. Consequently no evidence of any significant adrenergic mechanism affecting uterine secretion was obtained in these experiments.

## 3. Alterations in secretory responses after female sex hormone treatment, in estrous cycle and during pregnancy (I, III)

The experiments described above elucidating endometrial innervation and its sources were performed on animals purposely brought into artificial secretory phase. For natural reasons it appeared to be of interest to investigate possible secretory responses during normal reproductive cycle of the guinea-pig and after excess of estrogen and/or progesterone treatment.

*Combined treatment with estrogen and progesterone* a) Intact animals (I) treated with the commercial product Primocluton®. Field stimulation of nerves produced a secretory response of +71 %. Carbachol ( $5.5 \times 10^{-5}$  M) raised the secretion to the same level (+68 %). Threshold concentration was  $5.5 \times 10^{-6}$  M (see above).

b) Spayed animals (III) treated with the commercial products Progynon® (estrogen) and Pro-luton® (progesterone). Transmural stimulation of nerves increased secretion by 56 %. Carbachol produced an increase in secretion by about the same magnitude (+41 % at  $5.5 \times 10^{-5}$  M and +49 % at  $5.5 \times 10^{-4}$  M) as did transmural stimulation of nerves. The sensitivity to carbachol was similar to that of the intact animals treated with both hormones. (The differences between these two groups in degree of response to transmural stimulation or to carbachol are not statistically significant.)

*Single hormone treatment of spayed animals (III).* a) *Estrogen.* Field stimulation of nerves resulted in an increase of secretion by 14 % Carbachol ( $5.5 \times 10^{-6} M$ ) raised the secretion to about the same level as did nerve stimulation (+25 %). The response was shifted to the right by more than the power of ten, compared to the group receiving both estrogen and progesterone.

b) *Progesterone.* Neither field stimulation of nerves nor carbachol (up to  $5.5 \times 10^{-6} M$ ) produced any definite secretory responses in this group.

*Estrous cycle (III).* Responses to transmural nerve stimulation of about +20 % were noted around estrus (day 16—2) and at early diestrus (day 6—11). At metestrus (day 3—5) an inhibitory answer was obtained after field stimulation of nerves (—18 %). This response was reversed when phenolamine ( $1.6 \times 10^{-6} M$ ) was added (+25 %). However methoxamine ( $4 \times 10^{-6} M$ ) did not produce any definite effect on the secretion. The response to carbachol ( $5.5 \times 10^{-6} M$  —  $5.5 \times 10^{-4} M$ ) reached about +20 % at day 16—11 i.e. an answer of the same magnitude as that to transmural stimulation of nerves. The secretory responses of uteri from animals in estrous cycle (day 16—11) showed the same sensitivity to carbachol as those brought into artificial secretory phase by combined female sex hormone treatment. The magnitude of the response was however only about one third of the latter.

*Pregnancy (III).* At implantation time (day 6—8) a response of about +20 % was obtained by transmural stimulation of nerves as well as by carbachol ( $5.5 \times 10^{-6} M$  —  $5.5 \times 10^{-4} M$ ). From day 16—42 transmural stimulation was impossible to accomplish since the uterus wall was partly spayed (see Material and Methods III). At day 16—18 no secretory response to carbachol ( $5.5 \times 10^{-6} M$  —  $5.5 \times 10^{-4} M$ ) was obtained. At mid pregnancy an increasing response to carbachol ( $5.5 \times 10^{-6} M$  —  $5.5 \times 10^{-4} M$ ) was noted (+30 % at day 28—32, +50 % at day 38—42). In late pregnancy the response to carbachol ( $5.5 \times 10^{-6} M$ ) reached +62 % at day 48—52 and +56 % at day 63—65. The response to transmural stimulation of nerves was of a similar magnitude in late pregnancy as that seen after carbachol. The responses to transmural nerve stimulation and to carbachol of uteri in late pregnancy were of the same degree as those of uteri from animals brought into artificial secretory phase (cf. above). The sensitivity to carba-

chol seemed however to be somewhat lower *Post partum* the secretory response (about +50 %) after transmural nerve stimulation as well as to carbachol ( $5.5 \times 10^{-6} M$  —  $5.5 \times 10^{-4} M$ ) persisted.

Apparently carbachol raised the secretion to the same level as did transmural stimulation of nerves, when both stimuli were tested. However the magnitude of the response as well as the sensitivity to carbachol varied with the hormonal state of the animal. Combined treatment with estrogen and progesterone seems to mimic late pregnancy with respect to the magnitude of the responses. The response in estrous cycle (day 16—11) is smaller (about one third) but the sensitivity to carbachol is similar to that of uteri brought into artificial secretory phase. Uteri from animals treated with estrogen only also showed answers of smaller magnitude but were also less sensitive to carbachol.

Some aspects on these differences have to be mentioned. With respect to the magnitude of the responses one must keep in mind the variation of the resting secretion level. A high resting secretion level might more or less disguise a secretory response (cf. below III).

The sensitivity to carbachol was less in the group receiving estrogen only. Also pregnant uteri tended to be less sensitive to carbachol. From this it can be suggested that changes in the sensitivity of number of and accessibility to receptors can take place according to the hormonal state of organism. The absence of responses to both carbachol and transmural stimulation of nerves in late diestrus and of uteri from animals treated with progesterone only is probably explained by an inability of the autonomic cells to respond to secretory stimuli at least with respect to the parameters studied (see also below).

Obviously both female sex hormones seem to be necessary for development of full secretory effect. Female sex hormones are necessary for the development of the secretory cell i.e. the growth of organelles as mitochondria and Golgi apparatus as well as organization of microtubuli. These organelles seem to be necessary for secretory ability (Thermann & Schünke 1963, Dallenbach Hellweg 1969, Lawn 1973, Hafez & Ludwig 1977, Wynn 1977). Furthermore the development of the glands in secretory phase increases the area of mucus producing cells which can affect the secretory response as well as the resting



level. Moreover variation in type and amount of carbohydrate secreted may contribute to changes in the secretory responses and also to resting secretion level. Such changes in secretory products occur regularly in the human endometrium during reproductive cycle (Cramer & Klöns 1955, Runge Ebner & Lindenschmidt 1956, Schmidt Matthiesen 1963). In this context it should be recalled that endometrial secretion seems to be of the apocrine type (vide e.g. Wetzstein & Wagner 1960, Schmidt Matthiesen 1963, Hafez & Ludwig 1977). This type of secretion might be the reason for the all or none answer to secretory stimuli (cf. I).

In this context the serum levels of estrogen and progesterone in the reproductive cycle of the guinea pig should be mentioned. Concerning progesterone, the serum concentration is lowest during late diestrus (day 11–15) and reaches its peak in metestrus and early diestrus (day 5–9). The estrogen levels cannot be adequately measured during the estrous cycle (Feder Resko & Goy 1968, Challis, Heap & Illingworth 1971, Crox & Franchmont 1975). In mid and late pregnancy the progesterone concentration is 100-fold increased compared to the peak in estrous cycle. The estrogen levels increase from mid to late pregnancy about 3-fold (Heap & Deansley 1966, Challis et al. 1971). Accordingly the combined treatment with estrogen and progesterone chosen in this study seems to mimic the hormonal state at late pregnancy.

Possible alternative or contributive reasons for the variations in secretory responses could be hormonally caused changes in the neuroeffector region such as alterations in nerve fibre density, in transmitter content of nerve terminals, in size of junctional cleft and in the sensitivity number and distribution of postjunctional or maybe, also prejunctional receptors. Thus it has been reported that mucosal acetylcholinesterase positive nerves are more numerous and more intensely stained at estrus in the rat (Adham & Schenk 1969). It has also been suggested that endometrial nerves of the macaque degenerate at menstruation (Jacobson & Nieves 1961).

Since the magnitude of the responses to nerve stimulation and carbachol respectively essentially were parallel in all groups where secretory responses were obtained, and both stimuli could be applied, it seems unlikely that changes at least in the neural part of the region contributed very

much to the observed differences in size of responses. However concerning the absence of responses of uteri in late diestrus or dominated by progesterone only alterations as e.g. paucity of postjunctional receptors and degeneration of nerves might have some significance.

The results obtained with field stimulation at day 3–5 in estrous cycle indicated an  $\alpha$ -adrenergic, possibly neurogenic, inhibition of the secretory response. This inhibition could be pre or post junctional or both. The absence of a clear-cut effect of the exogenous  $\alpha$ -adrenoceptor agonist methoxamine (I III) on uterine secretion would suggest a prejunctional inhibition (Paton & Vizi 1969) (vide Burnstock & Costa 1975, Stjärne 1977). However one must also take possible diffusion barriers to exogenous agonists created e.g. by the outer layers of the endometrium into consideration. Therefore, at present, it is impossible to definitely point out the localization of the adrenergic inhibition. Adrenergic nerves in the endometrium are essentially localized in the basal endometrium (see Introduction). Also this localization might suggest a prejunctional site of action of these nerves on cholinergic secretomotor innervation. The fact that clear evidence of an adrenergic counteraction of the cholinergic response to field stimulation was noted only at day 3–5 in estrous cycle could be due to the circumstance that adrenergic innervation and noradrenaline content in the guinea-pig uterus are hormone dependent (vide III). It should however also be kept in mind that changes in stimulation frequencies might have revealed such an influence in other preparations too.

A further conclusion, possibly quite significant, can be drawn from the results i.e. there does not seem to be any disappearance of the cholinergic secretomotor innervation during pregnancy. This is in line with the results of Thorbert et al. (1979) showing that cholineacetyltransferase levels are not influenced during pregnancy. The statement made above concerning cholinergic endometrial innervation contrast to what happens to the myometrial adrenergic innervation during pregnancy which disappears (cf. Öwman et al. 1974, Thorbert 1978). Also combined treatment with estrogen and progesterone reduces uterine noradrenaline content (Thorbert et al. 1978b). The largest secretory responses to nerve stimulation were noted in uteri of animals receiving huge doses of these

hormones or being in late pregnancy. The neurogenic cholinergic secretory responses persisted post partum while still three months post partum it is difficult to discern adrenergic terminals (Thorbert et al. 1978a).

#### 4. Resting secretion level and endometrial carbohydrate concentration (I & III)

In this section the resting secretion levels of the different groups will be compared and commented upon. Since secretion level might depend on carbohydrate concentration of the mucosa, this will also be commented.

Resting secretion level per 10 min was rather uniform throughout all series with maximum occurring at pregnancy day 16–18 and in late pregnancy (day 63–68). The lowest values were seen in the groups receiving combined hormone treatment ( $0.34 \pm 0.14 \mu\text{mol}$   $n=49$  (I) and  $0.47 \pm 0.17 \mu\text{mol}$   $n=21$  (III)).

As mentioned above and in paper III a high resting secretion level might disguise a possible secretory response e.g. at pregnancy day 16–18. This possibility should also be considered in the case of lack of secretory responses in late diestrus and in the group treated with progesterone only. In estrous cycle the resting secretion level did not differ statistically between the groups ( $p > 0.05$ ) but the levels were always high ( $0.61 - 0.85 \mu\text{mol}$ ) (cf. Table I in paper III) as they were in the estrogen treated group ( $0.72 \mu\text{mol}$ ) and in the progesterone treated group ( $0.60 \mu\text{mol}$ ) (cf. Table III in paper III). Since secretory responses were

noted in estrous cycle (day 16–11) and in the estrogen treated group despite high resting secretion levels it seems unlikely that the inability to respond to secretory stimuli of uteri from the progesterone treated group and of uteri in late diestrus phase merely is an expression of a high resting secretion level masking a possible secretory response.

Carbohydrate leakage from all types of uterine tissue makes however the evaluation of secretory responses somewhat uncertain. For natural reasons the uncertainty is most marked when small secretory responses and small differences in secretory responses between groups are evaluated. This is one reason why one cannot make precise statements on e.g. the effect of methoxamine (I, III) phenolamine (I) and interruption of pelvic nerve or coxouterine connexion (I).

Throughout the series endometrial carbohydrate concentration was determined in order to obtain information of conceivable changes in carbohydrate concentration due to the hormonal state of the uterus (value for experiments in paper I  $0.34 \pm 0.13 \mu\text{mol/g}$   $n=49$  (previously unpublished) for others see III). However the endometrial carbohydrate concentration was rather uniform in all the series with maximum occurring in pregnancy day 16–18 and in late pregnancy (day 63–68) (III). This conformity is probably due to the circumstance that female sex hormones rather affect certain types of carbohydrates and their relative distribution in the glandular epithelial and stromal layers of the endometrium than change the total carbohydrate concentration (Schmidt Mathiesen 1963 III).

## IV GENERAL DISCUSSION

Uterine secretion and especially cervical secretion and the role of the mucus in mammalian reproduction have been subject to great interest for many years. Since now in addition to earlier morphological studies evidence exists of a functional secretomotor innervation of the endometrium of one species (the guinea pig) some brief speculations on the function of this innervation seem to be justified. Uterine innervation has been claimed to be insignificant since spinal cord lesions and section of the hypogastric or presacral nerve and the pelvic nerve of various species e.g. rabbit, dog and man do not seem to create an obstacle for fertilization, fetal growth and delivery in individual cases (Goltz & Frensborg 1874 Rein 1880 Genstmann 1926, Bacq & Brouha 1932, Wetherell 1933, Donaldson 1936, Cotte 1937 Pearce 1940, Blinkin 1947 Newell & Smithwick 1947 Poppen & Lemmon 1947 Gutman 1964 Jackson 1964 Hardy & Warrell 1965). However about half the uterine innervation is derived from short neurons, the cell bodies of which are located in the paracervical ganglia. This implies that at least the possible trophic effects of the nerves remain, after the above mentioned lesions. Furthermore, a controlled study on the effects of denervation on a large group of individuals has not been performed there are only case reports.

Female sex hormones constitute the necessary basis for uterine function and as far as uterine secretion is concerned the hormones cause the development of the secretory cells. This development is essential for the secretion of mucus from the cells (cf. Chapter III). The nerves may then act as fine modulators on the secretory cell.

The significance of the nerves could either be merely trophic or directly secretomotor which the present results suggest. Discharges in secretomotor nerves might arise on a purely reflexogenic basis, as a result of sensations due to external or internal stimuli, due to adjustments in central regulatory functions e.g. at the hypothalamic level or as a combination of these possibilities. In this context it should be mentioned that there may also occur inhibition of secretory impulses at various central and/or peripheral levels as e.g. on the effector cell

or in the autonomic ground plexus (cf. III). Reflexes could also be peripheral and local and relay in the inferior mesenteric ganglion (cf. Crowcroft, Holman & Szurzewski 1971) or maybe in the paracervical ganglia.

Uterine secretion is important under various conditions. The possibility that the secretion contains *pheromone(s)* has to be considered. It is however more likely that vulval secretions contain pheromones. Pheromone secretion of the guinea-pig supra-caudal sebaceous glands has been postulated (Paries 1960). Odorous substances are probably more significant for mammals other than man. Another possible function of the mucus would be as a *lubricant*. However secretions from the vagina and the vulval glands probably contribute to a greater extent to this function.

Uterine secretion is probably of great importance for *spermatozoal transport survival and capacitation*. The migration of spermatozoa from the vagina to the uterine cavity is dependent on the cervical secretion i.e. the amount as well as the type of mucus secreted. The migration is facilitated at ovulation and is prevented in late secretory phase (diestrus). The changes in secretion are evoked by female sex hormones, mainly estrogen (Homburger et al 1963 Schmidt Matthiesen 1963 Moghissi 1973a, b, Odeblad 1973 Hamner 1973 Chrétien 1974 1977). Both cervical and corporal secretion probably contributes to the survival of the spermatozoa (Chang 1967 Hamner 1973 Moghissi 1973b, Chrétien 1977). Cervical crypts act as reservoirs of spermatozoa and spermatozoal metabolism is increased in the uterine cavity (Gibbons & Mattner 1971 Moghissi 1971 Chrétien 1977). It is evident that capacitation of the spermatozoon requires exposure to uterine and/or tubal secretion (Chang 1967 Chang & Hunter 1975).

Further important roles for uterine secretion, mainly the corporeal, have been postulated such as *nourishment of the blastocyst* when it reaches the cavum uteri (Cartier Moricard & Moricard 1960 Shettles & Schmidt Matthiesen 1963 Chang 1967 Beler Petry & Kühnel 1970) and during implantation until a placenta is developed. For mammals uterine secretion could be of utmost importance.

Furthermore the secretory products may create an immunological barrier against spermatozoa and the fetus which could be considered as a homograft (Bradbury Billington & Kirby 1965 Bradbury et al 1969). Uterine secretion, mainly cervical secretion, is known to contain immunoglobulins and could thereby form a protection against microorganisms (Hulka & Omsrud 1969 Moghissi 1973b, Chrétien 1977).

Since during a great part of the reproductive cycle neurogenic secretory responses of the guinea-pig endometrium could be elicited, nervous modulation of uterine secretion could be of significance in various situations.

Regarding possible functions of the uterine secretion as a lubricant or as pheromone(s) increased firing in secretomotor fibres may occur e.g. estrus or during sexual excitement. Decreased neurogenic secretion could take place during e.g. anxiety and fright.

Neuronal discharges during sexual excitement may increase and change uterine mucus in such a way that transport, migration, survival and capacitation of spermatozoa are facilitated. It is also possible that copulation as well as deposition and/or entrance of sperm into different parts of the female genital tract may reflexively influence uterine secretion via secretomotor nerves. On the other hand, during unfavourable conditions neurogenic inhibition of uterine mucus secretion may prevent conception and might participate in regulation of animal population. In women such inhibition could be one possible cause of psychosomatic infertility.

Changes in uterine secretion, mainly the cor-poreal, due to secretomotor impulses elicited by adjustments in central regulation or due to reflexes may also facilitate nutrition of the blastocyst before, during and/or after implantation until placentalisation has been established. Such alterations in uterine mucus secretion, may also facilitate nidation. There is also the possibility that decreased cholinergic stimulation of uterine glandular cells caused by e.g. a peripheral adrenergic suppression of transmitter release may delay the time of maximum apocrine secretion until the blastocyst enters the uterine cavity hence reserving mucus for the crucial events. On the other hand a decreased muscarinic stimulation of mucus cells may inhibit secretion and thereby implantation.

Changes in uterine secretion due to secretomotor discharge may of course be of immunological significance with respect to the events leading to fertilization of the ovum as well as in the events leading to implantation and further development of the conceptus.

In conclusion, since uterine mucus is of great importance to mammalian reproduction, modulation of it caused by varying degrees of muscarinic stimulation of the secretory cells could be of significance for reproduction. As with many other factors influencing fertility the proper role of the secretomotor nerves may vary due to the situation and the species concerned. The work presented in this thesis points out that attention should be taken to secretomotor nerves whenever female fertility is considered.

## V SUMMARY

1 Secretory responses were measured as carbohydrate release from endometria of isolated everted uterine horns from guinea pigs. The presence of acetylcholinesterase positive nerves in the guinea pig endometrium was studied.

2. Transmural field stimulation produced an increase in carbohydrate release from uteri brought into artificial secretory phase by a combined treatment with an excess of estrogen and progesterone. The secretory response was abolished by tetrodotoxin and scopolamine but not by phentolamine or sotalol. Acetylcholinesterase positive nerves were found in the endometrium close to glandular cells.

3 The response to field stimulation was reduced after section and degeneration of the hypogastric nerves. It was essentially eliminated after destruction of the paracervical ganglia. Neither previous section of the pelvic nerves nor chronic interruption of the costouterine connexion produced any overt effect on the response. Stimulation of the hypogastric nerve but not stimulation of the pelvic nerve produced a definite increase in uterine carbohydrate secretion.

4 Carbachol but not methoxamine or isoprenaline produced a similar increase in uterine secretion as did field stimulation. The response to carbachol was blocked by scopolamine. Like the response to field stimulation the response to carbachol was of the all or none type and showed little if any gradation.

5 Uteri of spayed animals treated with merely estrogen responded to field stimulation and to carbachol but the answers were smaller and required higher concentrations of carbachol than those of uteri from animals treated with both estrogen and progesterone. Uteri from spayed

animals treated with merely progesterone did not respond to field stimulation or carbachol.

6. Except during the late diestrus phase, secretory responses to field stimulation and to carbachol could be elicited throughout the estrous cycle. The responses were of a smaller magnitude than those of uteri brought into artificial secretory phase. The sensitivity to carbachol was however the same. At metestrus (day 3-5) in estrous cycle evidence of an inhibitory possibly neurogenic, adrenergic influence on the secretory response to field stimulation was obtained.

7 Secretory responses to field stimulation and carbachol were noted at day of implantation in pregnancy. During pregnancy responses of increasing magnitude were obtained. In late pregnancy the response was of similar magnitude as that of uteri brought into artificial secretory phase. Secretory responses persisted post partum.

8 It is concluded that the guinea-pig endometrium possesses a cholinergic secretory innervation. The innervation seems to be essentially sympathetic with pre- and postganglionic fibres running in the hypogastric nerve. The former fibres relay on short neurons in the paracervical ganglia. The receptors on secretory cells are muscarinic.

9 It is further concluded that neurogenic secretory responses can be elicited during all the important stages of the reproductive cycle of the female guinea-pig. The all or none character of the secretory responses studied could be due to an apocrine type of secretion.

10. It is suggested that neurogenic modulation of uterine mucus secretion could be of importance for fertility. This possibility should be considered whenever mammalian fertility is discussed.

## VI ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to

Assistant Professor Nils O Sjöstrand my teacher for introducing me into the subject and for his generous support and interest of which he has supplied me throughout the study. His creativeness, vast knowledge and ability of constructive criticism have been of utmost importance to me. Our warm companionship, improving with many stimulating discussions during these years, has much facilitated the work.

Professor Bengt Andersson, head of the department, for generous support and advice.

Mrs Annika Rosén and Mrs Kerstin Westling, for all invaluable advice and skilful technical assistance throughout the study and for creating a pleasant and friendly atmosphere at the laboratories.

Mr Jan-Olof Hedborn, for his excellent assistance in the experiments.

Mrs Ann Hagström for supervising the determinations of the reproductive cycle of the animals, and to her and Mrs Ann-Marie Westerberg for taking excellent care of the animals.

Miss Gun Larsson and Miss Eva Svensson for expert and patient preparation of the manuscripts.

Mr Reijo Heikkilä and Mrs Annika Rosén for drawing the figures and to Mr Staffan Backman for taking the photographs.

Mr Jan Svensson for the skilful design of the electrodes and organ baths, and Mr Sven Karlsson for putting the cryostat in order.

Dr Naomi Clyne for revising the English.

Hans-Ake Hammarström, my husband, for his engagement in the work and his never failing support and patience.

The work was supported by grants from Harald and Greta Jeansson Stiftelse, Svenska Sällskapet för Medicinsk Forskning and Magnus Bergvalds Stiftelse. Schering AG generously provided the hormone preparations.

## VII REFERENCES

- ADHAM, N. & SCHENK, E. A. 1969 Autonomic innervation of the rat vagina, cervix and uterus and its cyclic variation. *Am J Obstet Gynec* 104: 508—516.
- BACQ, Z. M. & BROUHA L. 1932. Recherches sur la physiologie du système nerveux autonome II. Le comportement des organes génitaux après dénervation sympathique. *Arch Int Physiol* 25:250—285
- BASCH, S. v. & HOFMANN, E. 1877 Untersuchungen über die Innervation des Uterus und seiner Gefäße. *Med Jahrbücher* pp 465—487
- BEIER, H. M., PETRY G & KOHNEL, W. 1970. Endometrial secretion and early mammalian development. In *Mammalian Reproduction*. Eds. Gl. blau, H. & Plotz, pp. 264—285 E. J. Springer Verlag, Berlin-Heidelberg
- BELL, Ch. 1972. Autonomic nervous control of reproduction. Circulatory and other factors. *Pharmacol Rev* 24:657—736.
- BELL, Ch. 1974a. Selective cholinergic denervation of the uterine artery in the guinea-pig. *Experientia* 30:257—258.
- BELL, C. 1974b. Control of uterine blood flow in pregnancy. *Med Biol* 52:219—228
- BISHOFF T L. W. 1852 *Entwicklungsgeschichte des Menschen*. J. Richter'sche Buchhandlung, Gießen
- BLAND, K. P. & DONOVAN M. T. 1970. Oestrogen and progesterone and the function of the corpora lutea in the guinea-pig. *J Endocr* 47:225—230
- BLANDAU R. J. 1949 Observations on implantation of the guinea-pig ovum. *Anat Rec* 103:19—47
- BLINICK, G. 1947 Painless labors following pre-sacral neurectomy. *Am J Obstet Gynec* 53:148—151
- BOLING J L., BLANDAU R. J. WILSON J G & YOUNG W C. 1959 Post-parturitional heat responses of newborn and adult guinea pigs. Data on parturition. *Proc Soc exp Biol Med* 42:128—132.
- BRADBURY S BILLINGTON W D & KIRBY D R S. 1965 A histochemical and electron microscopic study of the fibrinoid of the mouse placenta. *J R Microsc Soc* 84:199—211
- BRADBURY S BILLINGTON W D KIRBY D R S. & WILLIAMS, E. A. 1969 Surf on mucin of human trophoblast. *Am J Obstet Gynec* 104:416—418.
- BROWN W H & HIRSCH E P. 1941 The intrinsic nerves of the immature human uterus. *Am J Path* 5:731—739
- BURGOS, M. H. & WISLOCKI, G. B. 1956. The cyclical changes in the guinea pig uterus, cervix vagina and sexual skin investigated by histological and histochemical means. *Endocrinology* 59:93—118.
- BURGOS, M. H. & WISLOCKI, G. B. 1958. The cyclical changes in the mucosa of the guinea pig's uterus, cervix and vagina and in the sexual skin, investigated by the electron microscope. *Endocrinology* 63:106—121
- BURN J H. 1938. Sympathetic vasodilator fibres. *Physiol Rev* 18:137—153
- BURNSTOCK, G. & COSTA, M. 1975 *Adrenergic Neurons, Their Organization, Function and Development in the Peripheral Nervous System*. Ch. 2, pp 4—18. Chapman and Hall, London.
- CARSTEN M E. 1968. Regulation of myometrial composition, growth, and activity. In: *Biology of Gestation*. Vol. I *The Maternal Organism*. Ed. Asall, N. S. Ch. 6, pp 356—425 Academic Press, New York & London.
- CARTIER, R., MORICARD F & MORICARD R. 1960 De l'activité sécrétoire de l'épithélium cylindrique utérin et d'un oedème du chorion cytogène précédant la nidation de l'œuf. In *Les fonctions de nidation utérine et leurs troubles*. pp 215—240 Masson et cie, Paris.
- CASAGLIA, G. & GIRO C. 1963 Morphologisch-funktionelle Aspekte des terminalen Nervensystem im Endometrium des menschlichen Fetus. *Zentralbl Gynäk* 32:1136—1142.
- CHALLIS, J R. G. HEAP R. B. & ILLINGWORTH, D V. 1971 Concentrations of oestrogen and progesterone in the plasma of nonpregnant, pregnant and lactating guinea-pigs. *J Endocr* 51:333—345
- CHANG M C. 1967 The reaction of the uterine endometrium on spermatozoa and eggs. In *Reproduction in the Female Mammal*. Eds. Lammung, G. E. & Asoroso, E. C. pp. 459—477 Butterworths, London
- CHANG M C & HUNTER, R. H. F. 1975 Capacitation of mammalian sperm: biological and experimental aspects. In: *Handbook of Physiology* Sect. 7 *Endocrinology* Vol. V *Male Reproductive System*. Eds. Hamilton D W & Greep, R. O. pp 339—351 American Physiological Society Washington.
- CHRÉTIEN F C. 1974 La glaire cervicale. II Propriétés chimiques et physiques — Ultrastructure. *J Gynec Obstet Biol Repr* 3:711—744
- CHRÉTIEN F C. 1977 La glaire cervicale. III Rôles physiologiques. *J Gynec Obstet Biol Repr* 6:451—488
- COTTE, G. 1937 Resection of the presacral nerve in the treatment of obstinate dysmenorrhea. *Am J Obstet Gynec* 33:1034—1040.
- COUJARD R. 1951 Quelques considérations sur le système nerveux autonome utéro-vaginal. *Gynec Obstet* 50:270—296.

- COUPLAND R. E. 1962. Histochemical observations on the distribution of cholinesterase in the human uterus. *J Obstet Gynaec Brit Emp* 69:1041—1043.
- COUPLAND, R. E. 1969. The distribution of cholinergic and other nerve fibers in the human uterus. *Postgrad Med J* 45 78—79.
- CRAMER, H. & KLÖSS, D. 1955. Verbesserung der Funktionsdiagnostik am Endometrium durch routinemäßige histochemische Darstellung der Polysaccharide. *Arch Gynaek* 185 739—758.
- CROIX, D. & FRANCHIMONT P. 1973. Changes in the serum levels of the gonadotropins, progesterone and estradiol during the estrous cycle of the guinea-pig. *Neuroendocrinology* 19:1—11.
- CROWCROFT P. J. HOLMAN, M. & SZURSZEWSKI, J. H. 1971. Excitatory input from the distal colon to the inferior mesenteric ganglion in the guinea-pig. *J Physiol (Lond)* 219 443—461.
- DAHL, W. 1916. Die Innervation der Weiblichen Genitalien. *Z Geburtsh Gynäk* 78:539—601.
- DAHL, W. 1924. Die Nervenversorgung der weiblichen Geschlechtsorgane. In: *Die Lebensnerven*. Ed. Müller L. R. pp. 339—356. Springer Verlag, Berlin.
- DALLENBACH, F. G. & VONDERLIN D. 1973. The Innervation of the human endometrium. *Arch Gynaek* 215:365—376.
- DALLENBACH-HELLWEG G. 1969. *Endometrium*. Springer Verlag, Berlin.
- DAVIS, A. A. 1933. The Innervation of the uterus. *J Obstet Gynaec Brit Emp* 40:481—497.
- DAVIS, A. A. 1934. The premenstrual nerve. *Br Med J* 2:1—6.
- DISKE, Z. & POPPER, H. 1976. Über eine neue kolometrischen Mikrobildungsmethode der Kohlenhydrat in Organen und Körperflüssigkeiten. *Biol. Zeitschrift* 175:371—411.
- DONALDSON M. 1936. The results of sympathetomy in dysmenorrhea. *Proc R Soc Med* 29:947—949.
- EL-BADAWI, A. & SCHENA, E. A. 1967. Histochemical methods for separate, consecutive and simultaneous demonstration of acetylcholinesterase and norepinephrine in cryostat sections. *J Histochem Cytochem* 15:580—588.
- EMRYS-ROBERTS, E. 1909/10. The embedding of the embryo guinea-pig in the uterine wall and its nutrition at the stage of development. *J Anat Physiol* 44 192—203.
- FEDER, H. H. RESKO, J. A. & GOY R. W. 1968. Progesterone concentrations in the arterial plasma of guinea-pigs during the estrous cycle. *J Endocr* 40:505—513.
- FELDMANN N. G. 1935. Experimentell-morphologische Studien der Innervation der weiblichen Geschlechtsorgane. *Arch Anat Histol Embryol* 14:571—582 (698—699).
- FELLNER, L. 1887. Ueber die Bewegungs- und Nervenversorgung des Uterus. *Zentralbl med Wiss* 5:258—59.
- FOLKOW B. & NEIL, E. 1971. *Circulation*. Oxford University Press, New York.
- FRANKENHAUSER, F. 1867. *Die Nerven der Gebärmutter*. Friedrich Mauke Verlag, Jena.
- FREEMAN A. 1946. The distribution of the nerves to the adult human uterus. *Am J Clin Path* 16: 117—123.
- GABELLA, G. 1976. The costo-uterine muscle of the guinea-pig: A smooth muscle attaching the uterus to the last rib. *Anat Embryol* 150:35—43.
- GAWRONSKY N. v. 1894. Über Verbreitung und Codigung der Nerven in den weiblichen Genitalien. *Arch Gynaek* 47:271—283.
- GENTES, M. L. 1902. Note sur les nerfs et les terminaisons nerveuses de l'utérus. *C r Mem Soc Biol* 54 425—427.
- GERSTMANN M. 1926. Über Uterusinnervation am Hand des Falles einer Geburt bei Querschnittslähmung. *Wechr Geb hefte Gynäk* 73 253—257.
- GIBBONS, R. A. & MATTNER, P. E. 1971. The chemical and physical characteristics of the cervical secretion and its role in reproductive physiology. In: *Pathways to Conception*. Ed. Sherman, A. J. Ch. II pp. 143—155. Charles C Thomas Publ. Springfield.
- GOLTZ, F. & FREUSBERG A. 1874. Über den Einfluss des Nervensystems auf die Vorgänge während der Schwangerschaft und des Gebrauchs. *Pflüg Arch* 9:552—565.
- GRUBER, C. M. 1933. The autonomic innervation of the genito-urinary system. *Physiol Rev* 13 497—609.
- GUTTMAN L. 1964. The married life of paraplegics and tetraplegics. *Paraplegia* 2:183—188.
- HAFAZ, E. S. E. & LUDWIG H. 1977. Scanning electron microscopy of the endometrium. In: *Biology of the Uterus*. Ed. Wyan, R. M. Ch. 10 pp. 309—340. Plenum Press, New York.
- HAMNER C. E. 1973. Physiology of sperm in the female reproductive tract. In: *The Regulation of Mammalian Reproduction*. Eds. Segal, S. J. Crozier R. Corfman, P. A. & Conditille, P. G. pp. 203—214. Charles C Thomas Publ. Springfield.
- HARDY A. G. & WARRELL, D. W. 1963. Pregnancy and labour in complete tetraplegia. *Paraplegia* 3:182—186.
- HEAP R. B. & DEANSLEY R. 1966. Progesterone in systemic blood and placenta of intact and ovariectomized pregnant guinea-pigs. *J Endocr* 34 417—423.
- HELLERMAN W. 1927. *Historische Studie zur Erforschung der Innervation des Uterus mit einem Anhang über ihre Darstellungsmethoden*. Inaugural-Dissertation. Ebering, Berlin.
- HOLT S. J. HOBBIER, E. E. & PAWAN G. L. S. 1960. Preservation of integrity of rat tissues for cytochemical staining purposes. *J natl phys biochem Cytol* 7:383—386.
- HOMBURGER, F. BERNFELD, P. TREIGER, A. GROSSMAN M. S. & HARPEL, P. 1963. Endometrial secretions. *Mucous Secretions* 106 683—691.
- HOOBKAMER, J. 1913. *Die Nerven der Gebärmutter*. *Arch Gynaek* 99:231—44.



- HULKA J F & OMRAN K. F 1969 The uterine cervix as a potential local antibody secretor *Am J Obstet Gynec* 104 440-442.
- ISHII O 1928. Observations on the sexual cycle of the guinea pig. *Biol Bull mar biol lab Woods Hole* 38:237-250
- JABONERO F 1953 Etudes sur l système nerveux génital périphérique. *Acta anat* 18:295-325
- JACKSON F E. 1964 Pregnancy complicated by quadriplegia. *Obstet Gynec* 23:620-621.
- JACOBSON H. N. & NIEYES O 1961 Intrinsic nerve fibres of the primate endometrium. *Expl Neurol* 4 180-187
- JORDAN S. M 1970. Adrenergic and cholinergic innervation of the reproductive tract and ovary in the guinea pig and rabbit. *J Physiol (Lond)* 210:115P-117P
- KANERVA, L 1972. Development, histochemistry and connections of the paracervical (Frankenhäuser) ganglion of the rat uterus. Thesis. *Acta Institut Anatomiol Universitatis Helsinkiensis. Suppl. 2.*
- KARNOVSKY M. J & ROOTS, L 1964 A direct coloring thiocholine method for cholinesterases. *J Histochem Cytochem* 12:219-221
- KAULLA, K. N. v. AIKAWA, J. K., BRUNS, D. & WIKLE, W. T 1957 Secretory function of the human uterine cervix. *Fert Ster* 8 444-454
- KILIAN F. M 1841 Die Nerven des Uterus. *Z Rationelle Med* 105 41-100.
- KOPPEL K 1950 Histologische Untersuchungen gebärenden Nervenversorgung des Uterus *Arch Gynaek* 177:354-391
- KÖRNER, T 1865 Anatomische und physiologische Untersuchungen über die Bewegungsnerven der Gebärmutter Studien des Physiologischen Institut Bresla 3:1-54
- KÖSTLIN R 1894 Die Nervenendigungen in den weiblichen Geschlechtsorganen. *Fortschr Med* 12 411-462
- KRANTZ, K. E 1959 Innervation of the human uterus. *Ann N Y Acad Sci* 75 770-784
- KÖHNEL, W. & BEIER, H. M 1976 Studies on the innervation of the endometrium *Cell Tiss Res* 167 577-586
- KUNTZ, A. 1947 *The Autonomic Nervous System*. Lea & Febiger Philadelphia
- LABHARDT A 1906 Das Verhalten des Nerven in der Substanz des Uterus. *Arch Gynaek* 80:135-211
- LANGLEY J. N 1891 On the course and connections of the secretory fibres supplying the sweat glands of the foot of the cat. *J Physiol (Lond)* 12 347-374
- LANGLEY J. N 1893 Further observations on the secretory and vaso-motor fibres of the foot of the cat, with notes on other sympathetic nerve fibres. *J Physiol (Lond)* 17 296-314
- LANGLEY J. N & ANDERSON H. K 1896b The innervation of the pelvic and adjoining viscera. Part IV The internal generative organs. *J Physiol (Lond)* 18:123-130.
- LANGLEY J. N & ANDERSON H. K 1896b The innervation of the pelvic and adjoining viscera. Part V Position of the nerve cells on the course of the efferent nerve fibres. *J Physiol (Lond)* 18 131-139
- LASSMAN G 1965 Die Nervenversorgung der Uterusschleimhaut. *Arch Gynaek* 200:500-509
- LAWN A. M. 1973. The ultrastructure of the endometrium during the sexual cycle. In: *Advances in Reproductive Physiology* Ed. Bishop M. V. H. Vol. 6, pp. 61-95 Academic Press, London.
- LEE, R. 1841 On the nervous ganglia of the uterus. *Phil Trans Roy Soc London* 2:269-275
- LEE, R 1858. Engravings of the ganglia and nerves of the uterus and heart. Churchill, London.
- MABUCHI K. 1924 Morphologische Studien über das Verhalten der Nerven in den weiblichen Geschlechtsorganen des Menschen mit besonderer Berücksichtigung der Veränderungen ihres Verhaltens während der Gravidität und Menstruation und im zunehmenden Alter. Anhang: Die Nerven in der Nabelschnur und Plazenta. *Mitt Med Fak d. Kaiserlichen Uni Tokyo* 31 385-495
- MARCO R. de. 1930. Il significato del liquido fiorentino dell'utero della cagna nel periodo di calore. *Arch Fisiol* 29:181-190
- MARSHALL, J. M. 1970. Adrenergic innervation of the female reproductive tract. *Anatomy physiology and pharmacology Rev Physiol Biochem Pharmacol* 62 6-67
- MOGHISSI K. S 1971 Sperm migration through cervical mucus. In: *Pathways to Conception*. Ed. Sherman, A. I pp 214-236 Charles C Thomas Publ Springfield.
- MOGHISSI K. S 1973a. Sperm migration through the human cervix. In: *Cervical Mucus in Human Reproduction* Eds Elstein, M. Moghissi, K. S. & Barth, R. Ch. 11 pp 128-152 Scriptor Copenhagen.
- MOGHISSI K. S. 1973b Composition and function of cervical secretion. In: *Handbook of Physiology* Section 7. Endocrinology Vol. 11.2 Female Reproductive System. Ed Greep, R. O Ch 31 pp 25-48. American Physiological Society Washington
- MORICARD, R. & GIRO C. 1958. Structure du système nerveux terminal du tractus génital féminin. *Annls Endocr* 19:648-697
- NEWELL, J. L. & SMITHWICK, R. H. 1947 Pregnancy following tubodoneal splanch (ectomy for essential and malignant hypertension associated with chronic pyelonephritis. *New Engl J Med* 236 851-858.
- NISHIMURA M. 1954 Innervation of uterus in the human embryo. *Arch Histol J* p 6:233-245.
- ODEBLAD E. 1973 Biophysical techniques of assessing cervical mucus and microstructure of cervical epithelium. In: *Cervical Mucus in Human Reproduction* Eds. Elstein, M., Moghissi, K. S. & Barth, R. Ch 5 pp 58-74 Scriptor Copenhagen

- OWMAN C. & SJOBERG N-O 1966. Adrenergic nerves in the female genital tract of the rabbit with remarks on cholinesterase-containing structures. *Z. Zellforsch* 74:182-197.
- OWMAN, Ch. — SJOBERG N-O & SJOSTRAND N. O 1974. Short adrenergic neurons, a peripheral neuroendocrine mechanism. In: *Amino Fluorescence Histochemistry* Ed. F. Jivara, M. & Tanaka, C. pp 47-66. Igaku Shoin Ltd., Tokyo.
- PARKES, A. D 1960. The role of odorous substances in mammalian reproduction. *J Reprod Fert* 1:312-314.
- PATENKO Th. 1880. Über die Nervenendungen in der Uterusinnenhaut des Menschen. *Zentralbl Gynäk* 4:64-66.
- PATON W D M & VIZI E. S 1969. The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig ileum longitudinal muscle strip. *Br J Pharmacol* 33:10-28.
- PEARCE, T. V 1940. Childbirth after prostatic neuroectomy. *B Med J* 1:87-88.
- PHOENIX, C. H 1970. Guinea pigs. 1. Reproduction and Breeding Techniques for laboratory animals. Ed. Hafez, E. S. E. Ch. 14 pp 244-257. Lea & Febiger Philadelphia.
- POPPEN J L & LEMMON C 1947. The surgical treatment of essential hypertension. *JAMA* 134:1-9.
- PRIBOR, H. C. 1951. Innervation of the uterus. *Anat Rec* 109:339.
- REIN G 1880. Beitrag zur Lehre der Innervation des Uterus. *Physi Arch* 23:68-84.
- REMINGTON R D & SCHORK, M. A 1970. Statistics with Applications to the Biological and Health Sciences. Prentice Hall Inc. Englewood Cliffs, N. J.
- REYNOLDS, S. R. 1949. Physiology of the Uterus. 2nd ed. Part VI. Innervation of the Uterus. pp. 463-502. Hoeber Inc. New York.
- REYNOLDS, S. R. M 1965. Physiology of the Uterus. 3d ed. Part VI. Innervation of the Uterus. pp. 463-502. Hoeber Inc., New York.
- RUNGE, H. EBNER, H. & LINDENSCHMIDT W 1956. Vorräte der kohligen Alkanblau — Perjodsaure — Schiff — Reaktion für die gynäkologische Histopathologie. *Dtsch med Wochr* 38:1523-1529.
- ROSSE, H. W & MARSHALL, J. M 1970. Uterine response to adrenergic nerve stimulation in the guinea-pig. *Biol Reprod* 3:13-22.
- SANSONI, G. S. & HILL, J. P 1931. Observations on the structure and mode of implantation of the blastocyst of canis. *Trans Zool Soc* 21:295-334.
- SCHMIDT MATTHIESEN H 1963. Histochemie in: *Das normale menschliche Endometrium* Ed. Schmidt-Matthiesen, H. pp 149-244. Thieme Verlag, Stuttgart.
- SCHOFIELD, H. M 1952. The innervation of the cervix and corpus uteri in the rabbit. *J Physiol. (Lond)* 117:317-328.
- SCHRÖDER, R. 1930. Die weibliche Genitalorgane. In: *Handbuch der mikroskopischen Anatomie des Menschen*. VII/1 Harn- und Geschlechtsapparat. Eds. Mollendorf W v & Schröder R. pp 329-556. Springer Verlag, Berlin.
- SELLE, R. M 1922. Changes in the vaginal epithelium of the guinea-pig during the oestrous cycle. *Am J Anat* 30:429-449.
- SHABANAH, E. H. TOOTH, A. & MAUGHAN, G. B 1964. The role of the autonomic nervous system in uterine contractility and blood flow. I. The interaction between neurohormones and sex steroids in the intact and isolated uterus. *Am J Obstet Gynec* 89:841-859.
- SHETTLES, L. B. 1951. Polysaccharides in cervical mucus. *Fert Ster* 2:361-368.
- SHETTLES, L. B. DISHE, Z. & OSNOS, M 1951. Neutral polysaccharides of the human cervical mucus. *J Biol Chem* 192:589-593.
- SHETTLES, L. B. & SCHMIDT MATTHIESEN H. 1963. Endometrium and Nidation. In: *Das normale menschliche Endometrium*. Ed. Schmidt-Matthiesen, H. pp 294-316. Thieme Verlag, Stuttgart.
- SHIH, H. E., KENNEDY J & HUGGINS, C. 1940. Chemical composition of uterine secretions. *Am J Physiol* 130:287-291.
- SJOBERG, N-O 1967. The adrenergic transmitter of the female reproductive tract: distribution and functional changes. *Acta Physiol Scand* 72. Suppl. 305, 1-32.
- SJOQVIST F 1963. The correlation between the occurrence and localization of acetylcholinesterase-rich cell bodies in the stellate ganglion and the outflow of cholinergic sweat secretory fibres to the fore paw of the cat. *Acta Physiol Scand* 57:339-351.
- SJOSTRAND N O 1976. Le système nerveux et le tractus génital mâle. In: *Système nerveux, activité sexuelle et reproduction*. Eds. Soulaire, A., Gossard, J. P. Rouvenau, J.-P. & Cohen, J. pp. 183-190. Masson, Paris.
- SJOSTRAND N O & KLINGE, E. 1979. Principal mechanisms controlling penile retraction and protrusion in rabbits. *Acta Physiol Scand* 106:199-214.
- SPEE, F. Graf v 1901. Die Implantation des Meer-schwämmeis in die Uteruswand. *Z. Morph Anthr* 3:130-182.
- SQUIER, R. R. 1932. The living egg and early stages of its development in the guinea pig. *Contr Embryol* 137. Carnegie Inst. Washington, publ. 433 pp 223-250.
- STATE, D. & HIRSCH, E. F 1941. Nerves of the adult human endometrium. *Arch Path* 52:939-950.
- STJARNE, L. 1977. Basic mechanisms of local feedback control of secretion of adrenergic and cholinergic neurotransmitters. In: *Handbook of psychopharmacology* Eds. Iversen, L. L. & D. Snyder S. H. Vol. 6, pp. 179-231. Publ. Comp New York.

- STOCKARD C. R. & PAPANICOLAOU G. H. 1917 The existence of typical oestrous cycle in the guinea pig — with a study of its histological and physiological changes. *Am J Anat* 22:225—283
- STOCKARD C. R. & PAPANICOLAOU G. N. 1919 The vaginal closure membrane and the vaginal plug in the guinea-pig with further considerations of the oestrous rhythm. *Biol Bull mar Biol Lab Woods Hole* 37:222—245
- THEMANN H. & SCHÖNKE, W. 1963. Die Feinstruktur der Drüsenepithelien des menschlichen Endometriums. Elektronenoptische Morphologie. In: *Das normale menschliche Endometrium* Ed Schmidt Matthieson, H. pp 111—148. G Thieme Verlag, Stuttgart.
- THEOBALD G. W. 1973 Endocrine control of the uterine innervation. *Ch. 2*, pp 15—66. Butterworths & Co, London.
- THORBERT G. 1978. Regional changes in structure and function of adrenergic nerves in guinea pig uterus during pregnancy. *Acta Obstet Gynec Scand Suppl.* 79: 1—3
- THORBERT G. ALM, P. BJÖRKLUND A. & ÖWMAN C. & SJÖBERG N.-O. 1979 Adrenergic innervation of the human uterus. Disappearance of the transmitter and transmitterforming enzymes during pregnancy. *Am J Obstet Gynec.* 135:223—226.
- THORBERT G. ALM, P. ÖWMAN C. & SJÖBERG, N.-O. 1977 Regional distribution of autonomic nerves in guinea-pig uterus. *Am J Physiol* 233:C25—C34
- THORBERT G. ALM, P. ÖWMAN Ch. SJÖBERG N.-O. & SPÖRRONG B. 1978a. Regional changes in structural and functional integrity of myometrial adrenergic nerves in pregnant guinea pig and their relationship to the localization of the conceptus. *Acta Physiol Scand* 103:120—131
- THORBERT G. ALM, P. & ROSENGREN E. 1978b. Cyclic and steroid-induced changes in adrenergic neurotransmitter level of guinea-pig uterus. *Acta Obstet Gynec Scand* 57:45—48.
- THORBURN H. D. CHALLIS, J. R. G. & ROBINSON J. S. 1977 Endocrine control of parturition. In: *Biology of the Uterus*. Ed. Wynn, R. M. Ch. 17 pp 633—698. Plenum Press, New York.
- UVNÄS, B. 1960. Sympathetic vasodilator system and blood flow. *Physiol Rev* 40:69—80
- VARAGIC, V. 1956 An isolated rabbit hypogastric nerve-uterus preparation with observations on the hypogastric transmitter. *J Physiol (Lond)* 132:92—99
- WETHERELL, F. S. 1935 Intractable dysmenorrhoea. *Am J Obstet Gynec* 29:334—340
- WETZSTEIN R. & WAGNER, H. 1960. Elektronenmikroskopische Untersuchungen am menschlichen Endometrium. *Anat Anz* 108:362—375
- WYNN R. M. 1977 Histology and ultrastructure of the human endometrium. In: *Biology of the Uterus*. Ed Wynn, R. M. Ch. 11, pp 341—376 Plenum Press, New York.
- YOUNG W. G. 1936/37 The vaginal smears picture, sexual receptivity and the time of ovulation in the guinea pig. *Anat Rec* 67:305—325





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 405

## THE VAGAL CONTROL OF THE PYLORIC

A physiological and immunological study  
in cat and man

BY

ROGER EDEN



**ACTA PHYSIOLOGICA SCANDINAVICA**  
**SUPPLEMENTUM 485**

**FROM THE DEPARTMENT OF SURGERY III AND THE DEPARTMENTS OF HISTOLOGY  
UNIVERSITY OF GÖTEBORG AND KAROLINSKA INSTITUTE, STOCKHOLM, SWEDEN**

**THE VAGAL CONTROL OF THE PYLORIC MOTOR FUNCTION**

**A physiological and immunohistochemical study  
in cat and man**

**BY**  
**ROGER EDIN**

**GÖTEBORG 1980**



Distributör: Almqvist & Wiksell Tryckeri AB Rapsgatan 8 S-751 81 Uppsala, Sweden

FROM THE DEPARTMENT OF SURGERY III AND THE DEPARTMENTS OF HISTOLOGY  
UNIVERSITY OF GÖTEBORG AND KAROLINSKA INSTITUTE, STOCKHOLM SWEDEN

## ABSTRACT

**The vagal control of the pyloric motor function. A physiological and immunohistochemical study in cat and man.**

Roger Edin, Department of Surgery III, Sahlgren's Hospital, University of Göteborg, S 413 45 Göteborg, Sweden.

In acute experiments in anesthetized cats ( $n = 75$ ) with ligated adrenals the influence on the pyloric sphincter of afferent and efferent electrical stimulation of the cervical vagi was studied with a flow recording technique. Gastric motility was recorded simultaneously with a volume recording technique. *Efferent* stimulation using high threshold parameters (8 V, 5 ms, 8 Hz) resulted in a prompt gastric contraction and delayed pyloric contraction ( $23 \pm 17$  s). In one third of the cats *biphasic* pyloric motor response consisting of a short period of increased flow preceding a longlasting cessation of the flow was observed. When the transpyloric flow was reduced by splanchnic nerve stimulation or noradrenalin infusion, efferent vagal nerve stimulation induced an increased flow supporting the assumption of relaxatory fibres to the pylorus within the vagus as well. Unilateral *afferent* stimulation resulted in decreased flow within  $5 \pm 3$  s and a prompt gastric relaxation, which were both abolished at transection of the intact contralateral vagus indicating vago-vagal reflexes.

After atropine (0.2 mg/kg i. b.w.) *efferent* stimulation resulted in gastric relaxation, while the *biphasic* pyloric motor response was even more pronounced with a long (60 s) latency of the contractile phase. Addition of guanethidine (2 mg/kg i. b.w.) did not affect these responses. After hexamethonium (25 mg i.v. + 50 mg  $\pm$  10 mg i.a./h) the stimulation procedure resulted in a gastric relaxation, while the pyloric contraction was blocked. The relaxatory phase required the addition of atropine for blockade indicating separate transmission mechanisms for the two components of the pyloric motor response at such stimulation. Hexamethonium effectively antagonized the pyloric contraction at *afferent* stimulation.

Immunohistochemical studies revealed rich enkephalinergic innervation of the pylorus and the presence of enkephalin-like material within vagal axons. VIP-like material was demonstrated within the same tissues with a similar distribution. The hypothesis of transmission via non-classical receptors was corroborated in experiments, where the pyloric contraction at vagal *efferent* stimulation was blocked dose-dependently by the opiate receptor antagonist, naloxone i.a. Furthermore micromolar doses i.a. of enkephalins mimicked the vagal motor effects, i.e. pyloric contraction and gastric relaxation, which were reversed by equimolar doses of naloxone in favour of a *pyloric* *enkephalinergic* contraction. Intrarterial injection of nanomolar doses of this VIP resulted in both a gastric and pyloric relaxation.

Specimens of the human vagus from the thoracic and abdominal levels were investigated with immunohistochemistry. Substance P and enkephalin containing fibres and a low number of VIP fibres were seen at all levels indicating the presence of an axonal transport of these peptides in the human vagus.

**Key words:** Vagal nerve, electrical stimulation, neural transmission, immunohistochemistry, peptides, motor activity, pylorus, stomach, cat and man.

# CONTENTS

INTRODUCTION	1
AIM OF THE INVESTIGATION	3
MATERIAL AND METHODS	3
Physiological experiments	3
Operative procedure	3
Recording of transpyloric flow	5
Recording of gastric tone	5
Nerve operations and stimulation procedures	5
Drug infusion	6
Drugs	6
Immunohistochemistry	6
Preparation and characteristics of antisera	6
Immunohistochemical procedure	7
Collection of vagal nerves	7
METHODOLOGICAL CONSIDERATIONS	8
RESULTS	9
Physiological experiments on vagal neurotransmission by classical receptors	9
Efferent vagal nerve stimulation	9
Efferent vagal nerve stimulation during concomitant splanchnic nerve stimulation or noradrenalin-infusion	9
Efferent vagal nerve stimulation after pretreatment with atropine, guanethidine and hexamethonium	9
Afferent vagal nerve stimulation	11
Afferent vagal nerve stimulation after pretreatment with atropine, guanethidine and hexamethonium	12
Immunohistochemistry of peptides in the vagus and the pylorus	12
Immunohistochemistry of the feline pylorus and stomach	12
Substance P, VIP and enkephalin-like immunoreactivity in the human vagus nerve	12
Physiological experiments on vagal neurotransmission by non-classical receptors	14
Vagal nerve stimulation before and after naloxone and/or atropine	14

Intraarterial injections of morphine met and leu-enkephalin and naloxone	15
Intraarterial injections of VIP	16
DISCUSSION	16
Physiological experiments on vagal neurotransmission by classical receptors	16
Immunohistochemistry of peptides in the vagus and the pylorus of the cat	18
Physiological experiments on vagal neurotransmission by non-classical receptors	19
Immunohistochemistry of human vagal nerve	21
FUNCTIONAL CONSIDERATIONS	22
GENERAL CONCLUSIONS	23
ACKNOWLEDGEMENTS	24
REFERENCES	25

The present summary is based on the following publications:

- I The vagal control of the feline pyloric sphincter  
Edin R., H. Ahlman and J Kewenter  
*Acta physiol. scand.* 1979 107 169-174
- II The transmission mechanism of the vagal control of the feline pylorus.  
Edin R., H. Ahlman, A. Dahlström and J Kewenter  
*J Neur Transm.* 1980, Accepted.
- III Evidence for vagal enkephalinergic neural control of the feline pylorus and stomach.  
Edin R. J Lundberg, L. Terenius, A. Dahlström, T Hökfelt J Kewenter and H. Ahlman.  
*Gastroenterology* 1980 In press.
- IV On the VIP-ergic innervation of the feline pylorus.  
Edin R. J Lundberg, H. Ahlman, A. Dahlström J Fahrenkrug T Hökfelt and J Kewenter.  
*Acta physiol. scand.* 1979 107 185-187
- V Substance P VIP and enkephalinlike immunoreactivity in the human vagus nerve.  
Lundberg, J., T Hökfelt, J Kewenter G Pettersson, H. Ahlman R. Edin, A. Dahlström, G Nilsson, L. Terenius, K. Uvnäs-Wallensten and S Said  
*Gastroenterology* 1979 77 468-471

These papers will be referred to in the text by their Roman numerals.

## ABBREVIATIONS

ACh	=	Acetylcholine
Mo	=	Morphine
NA	=	Noradrenalin
A	=	Adrenalin
Atr	=	Atropine
Gua	=	Guanethidine
Hex	=	Hexamethonium
Nal	=	Naloxone
VIP	=	Vasoactive intestinal polypeptide
ENK	=	Enkephalin
SOM	=	Somatostatin
SP	=	Substance P
b w	=	body weight
i.a.	=	intraarterial
i.v.	=	intravenous

## INTRODUCTION

The gastroduodenal junction was named the pylorus (from the Greek *pylo-gate* - *ouras* - *warder-pylorus*) since it was believed to be the gateway to the intestine. The pylorus has been considered a separate functional entity subject to a special extrinsic innervation and reflex activation (Gaskell, 1916). In accordance with this view the vagus was at first considered to give only motor innervation to the pyloric region (Openchowaki, 1889). Later Agostoni et al. (1957) demonstrated that most of the subdiaphragmatical vagal axons were sensory and unmyelinated. Cunningham (1906) pointed out two components of the pylorus: One for closing (m. sphincter) and one for opening (m. dilatator) the lumen.

Anatomically the pylorus is well defined in most species by its conspicuous thick circular muscle layer forming two thirds of the thickness of the wall of the pylorus (Riddinger 1879 Torgersen, 1942). The sphincter component of the pylorus is formed mainly by the circular muscle layer and probably also by helical fibres, which are longitudinal in the stomach and more circularly arranged around the pyloric orifice (Didlo-Andersson, 1968). The dilatator component is composed of longitudinal muscle fibres (Belding & Kernohan, 1953), which are external to and penetrating into the circular muscle layer (Horton, 1928 Müller 1963). The longitudinal muscle fibres form one third of the wall and are intertwined with the circular muscle fibres of the antrum and inserted into the submucosal connective tissue of the gastroduodenal junction. A few superficial longitudinal muscle fibres are continuous with the corresponding duodenal muscle layer.

From the literature the conclusion may be drawn that the vagi convey both excitatory and inhibitory motor responses not only to the stomach but also to the pylorus (Katz & Winkler 1902 Cannon, 1906 Thomas & Wheelon 1922 Martinson & Muren 1963). A vagal inhibitory innervation of the pylorus was first suggested by Langley (1898) and later confirmed by May (1904). Subsequent work tended to discredit the idea of a special nervous control of the pylorus and rather established the view of pylorus as the last and most vigorous part of the antro-pyloric pump mainly participating in locally generated peristaltic activity (Edwards & Rowlands, 1968). In 1965 a high pressure zone of the gastroduodenal junction in dogs was demonstrated by Brink et al. (1965). Later this finding was confirmed in man using similar manometric techniques by Fisher & Cohen (1973) who demonstrated an increased intrapyloric pressure after duodenal perfusion with hydrochloric acid, olive oil or amino acids, and a decreased pressure at antral peristalsis. The physiological characteristics of a gastrointestinal sphincter proposed by Cohen et al. (1968) and Fisher & Cohen (1973) were thus fulfilled for the pylorus and expressed as follows

- a) A sphincter region which exhibits an intraluminal pressure greater than that of the cavities separated by the sphincter
- b) Appropriate stimulation proximal to the sphincter results in a consistent fall in the pressure within the sphincteric zone

- c) Appropriate stimulation distal to the sphincter results in a prompt rise in sphincter pressure

*In vitro* studies with electrical field stimulation (Anuras et al., 1974) in opossum, cat, dog and man suggest an inhibitory innervation of pyloric muscle cells. Efferent vagal nerve stimulation in dogs pretreated with atropine causes an inhibition of spontaneous pyloric motor activity indicating an atropine-resistant inhibitory innervation of the pyloric region (Mir et al., 1979). It has also been shown that the relaxation and "off motor response" in the pylorus in dogs following vagal nerve stimulation are nerve-mediated, since tetrodotoxin blocks these responses (Telford et al., 1979).

The integrated motor function of the stomach is very complex. It acts as a receptacle for food and as a digestive organ by converting solid food into chyme which is successively emptied into the duodenum. This is an oversimplification since food is emptied both as in liquid and solid form after a meal. The solid particles are emptied by peristalsis of the antral part of the stomach. Liquids or chyme do not depend on peristalsis in order to be emptied but on slow tonic pressure changes in the fundus-corpus part of the stomach (Code & Carlson, 1968; Mroz & Kelly 1977). The antral peristalsis has a speed of 3 cycles per minute in man, and vagal activation increases the amplitude of the peristaltic waves rather than changes their speed (Edwards & Rowlands 1968). There is also a retro-pulsion of solid particles, which means that food returns from the distal antrum to the fundus-corpus part of the stomach. The pylorus has also been ascribed a role in this process (Carlson et al., 1966).

It was noted early that the gastric emptying of various liquid meals is faster when a high duodenal fistula is allowed to drain the stomach preventing the gastric contents from contact with the duodenal mucosa (Thomas et al. 1934) indicating the existence of a "duodenal brake mechanism". In patients after a partial gastrectomy emptying has been shown to be approximately twice as rapid as in controls (Berger 1969). Furthermore the gastric emptying rate was found to be increased in patients where resection was combined with pyloroplasty and vagotomy as well (McGill et al., 1969).

The pyloric sphincter has another important action in preventing bile reflux from the duodenum into the stomach (Flaher & Cohen 1973; Valenzuela, 1976) a mechanism which may be important in the pathogenesis of peptic ulcer as pointed out by DuPlessis (1965). Because of the late complications of gastric resections such as the dumping syndrome and the development of carcinoma in the resected stomach, most surgeons favour highly selective vagotomies rather than gastric resections in order to save the pyloric function. The motor control of the pylorus may be exerted by several factors such as luminal stimulants, gastrointestinal hormones and the intrinsic and extrinsic nervous supply of the gastroduodenal region. The aim of the present study was to evaluate the role of the extrinsic vagal control of the pylorus in an experimental model in cats.

## AIM OF THE INVESTIGATION

The gastroduodenal junction is protected by a sphincter which fulfills the described characteristics of such a sphincter (1). Only a few studies have dealt with the extrinsic nervous control of the pyloric sphincter and the results are conflicting. Furthermore in addition to the classical transmitters NA and ACh, there is now evidence that other newly discovered transmitter substances, such as small peptides are to be found in the vagus nerve (Lundberg et al. 1978 a, b). These substances may serve as transmitters (cf. Otsuka & Takahashi 1977, Fahrenkrug et al., 1979) involved in a vagal control of the pyloric sphincter. The aim of the present investigation was therefore

- 1 To study *in vivo* the effects of vagal nerve stimulation on an externally applied flow of saline through the feline pyloric sphincter by means of a flow recording method. Furthermore gastric motility was studied simultaneously in order to investigate whether the vagal nerves might exert specific effects on the pyloric region separate from those on the stomach. To study the effects of cholinergic, adrenergic and ganglionic blocking agents on the pyloric motor responses obtained at efferent and afferent electrical stimulation.
- 2 To study by immunohistochemistry the possible occurrence of possible neurotransmitters within neurons of the gastric or pyloric wall of the cat.
- 3 To study the physiological effects of possible neurotransmitters, such as peptides localized to intramural neurons on pyloric and gastric motility *in vivo* in cats when administered locally via the splenic artery.
- 4 To demonstrate the existence of possible neurotransmitters, other than ACh and NA, in the vagal nerve in man by immunohistochemistry.

## MATERIAL AND METHODS

### Physiological experiments

#### Operative procedure

Experiments were performed on 75 adult cats of both sexes. The number of animals used in the various series is given in each paper. A few cats were used for experiments in several different series. The animals were fasted for 24 hours but had full access to water. Anaesthesia was initiated with ether and continued with chloralose (50 mg/kg b.w.) slowly injected through one of the femoral veins. A tracheal cannula was inserted to maintain a free airway and all animals breathed spontaneously except in a few experiments in which a ventilator was used. The animals were placed on an operating table with a thermostatically regulated heating pad in order to maintain the body temperature at about + 38°C. A femoral artery was cannulated and connected to a pressure transducer (Statham p 23 DC) for continuous recording of arterial blood pressure.

The abdomen was opened with a midline incision. Since catecholamine secretion from



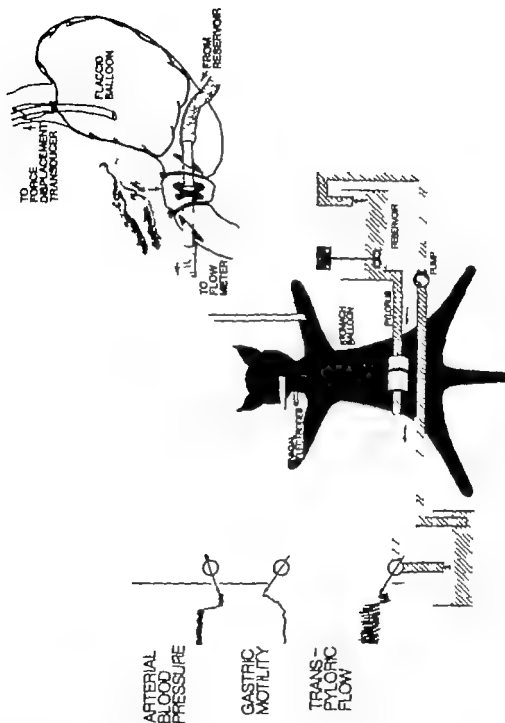


Fig. 1 Experimental model to study gastric and pyloric motility simultaneously in the anesthetized cat (for details, see text). *Inset* Detail of the set-up to study the transpyloric flow. Glass tubes separated only by the sphincter itself (see histology section).

the adrenal medulla is known to be a potent intestinal inhibitor (Kock, 1959) the vessels of these glands were ligated at the beginning of all experiments. The duration of each experiment was less than 4 hours and corticosteroid substitution was therefore not given.

#### Recording of transpyloric flow

A non-compressible plastic tube (diameter 8 mm) was inserted through a small longitudinal incision along the greater curvature of the anterior wall of the lower corpus region of the stomach and secured with a ligature immediately proximal to the pylorus. Consequently antral motor activity could not influence the transpyloric flow. A similar plastic tube was inserted through a longitudinal incision in the descending part of the duodenum and brought up to the pylorus just opposite the proximal tube. In this position the distal tube was secured with a ligature around the duodenum (Fig. 1)

A reservoir containing physiological saline and thermostatically regulated to 38°C, was connected to the proximal tube. The distal tube was connected to a photoelectric flow meter which recorded volume changes of the transpyloric flow between 20 – 1000 ml/min. The saline from the flowmeter was collected and constantly pumped back to the perfusion reservoir. Since the fluid surface of this reservoir was large (370 cm<sup>2</sup>), the inflow pressure through the sphincter could be kept almost constant recirculating the saline. The pressure gradient between the two reservoirs and the pylorus could be varied by adjusting the level in the reservoirs. The average height between the reservoirs was 50 cm. To initiate the inflow of saline through the pylorus a pressure of 20 cm H<sub>2</sub>O was induced and then reduced to about 10 cm H<sub>2</sub>O (Fig. 1)

#### Recording of gastric tone

A deflated plastic balloon of about 1000 ml volume was introduced orally into the corpus-fundus part of the stomach of the anaesthetized animal. The balloon was connected by a rubber tube to a water tank mounted on a weight recorder (Grass Force Displacement Transducer FT 10 C) and connected to an amplifier. The water level in the tank could be varied by changing the level of the weight transducer. By opening the system with the tank at different water levels in relation to the stomach, water was allowed to fill the gastric balloon. In most experiments such a hydrostatic pressure gradient was accomplished at 5 cm H<sub>2</sub>O. Blood pressure, intragastric tone and transpyloric flow were simultaneously recorded by means of a Grass polygraph (Grass S4 E) (Fig. 1)

#### Nerve operations and stimulation procedures

The cervical vagal nerves were isolated through the tracheostomy incision from the sympathetic trunk and divided uni- or bilaterally. The proximal and distal cut ends of the vagus were inserted separately into insulated ring electrodes, which were placed deep into the wound before nerve stimulation. In 16 cats, given *efferent* vagal nerve stimulation, the splanchnic nerves were left intact in 5 animals and in 11 animals divided bilaterally beneath the diaphragm at the preganglionic level before stimulation.

In animals where the unilateral *afferent* vagal nerve was stimulated the splanchnic nerves were left intact in 7 and divided bilaterally in 11 cats. In 4 animals both the splanchnic and contralateral vagal nerves were divided during the experiment. All nerve stimulations were performed using square wave pulses from a Grass stimulator (S4 E) over 1–10 min. The stimulation parameters (8–15 V 0.2–5 ms 8–12 Hz) were divided into 2 groups activating *high* (5 ms) and *low* threshold (0.2 ms) fibres selectively according to Martinson (1965).

### Drug infusion

A fine plastic catheter (diameter 0.5 mm) was introduced via the splenic artery in a retrograde direction and the tip of the catheter placed at the junction with the celiac artery. The infusion of ink through this catheter after the experiments showed a distribution to the stomach, pylorus, and proximal part of the duodenum. The substances were dissolved in saline at body temperature before the infusion.

### Drugs

Atr (Atropine sulfate Merck 0.1–0.2 mg/kg) and Gua (Guanethidine sulfate CIBA 2 mg/kg) were given i.v. Hex (Hexamethonium chloride Fluka AG) was given as a bolus dose i.v. (25 mg/kg) followed by an i.a. infusion ( $50 \pm 10$  mg/kg b.w. hr). Mo (Morphine chloride ACO Sweden, 0.1–1 mg; 0.3–3.1  $\mu$ mol) (D-Ala<sub>2</sub> met<sup>5</sup>)-enkephalinamide (Bachem, Bubendorf Switzerland, 0.2–1 mg, 0.3–1.32  $\mu$ mol) [met<sup>5</sup>]- and [leu<sup>5</sup>] enkephalin (Bachem Switzerland) (0.05–0.01 mg; 0.08–0.16  $\mu$ mol) ACh (Acetylcholine chloride Roche 0.05–0.1 mg, 0.3–0.6  $\mu$ mol) VIP (generously supplied by Professor Viktor Mutt Dept of Biochemistry Karolinska Institute Stockholm Sweden, 0.1–4  $\mu$ g; 0.03–1.3 nmol) were given as bolus doses i.a. Nal (Naloxone chloride Endo) was given at an infusion rate of 0.1–0.4 mg/kg b.w. min at a total dose of 2.6–6 mg (6.9–16.6  $\mu$ mol) or as a bolus dose of 0.1–0.4 mg (0.3–1.2  $\mu$ mol).

## Immunohistochemistry

### Preparation and characteristics of antisera

All antisera were raised in rabbits using SP VIP and met ENK conjugated to bovine serum albumin. Characterization of these antisera revealed that the SP antiserum (K25) crossreacts to a negligible extent (<0.1 %) with ENK, VIP, SOM, glucagon, bradykinin, and edetoxin (Nilsson et al., 1975). The VIP antiserum (5603–5) shows negligible cross-reactivity (<0.1 %) with secretin, glucagon, cholecystokinin-pancreozymin, pancreatic polypeptide, SP, SOM, neurotensin,  $\alpha$ -endorphin, leu and met ENK, bradykinin and angiotensin (Fahrenkrug et al. 1977). Finally the met ENK antiserum (336) shows negligible crossreactivity (<0.1 %) with the large molecular weight endorphins, SP, VIP and SOM but crossreacts to about 25 % with leu-ENK (Schultzberg et al., 1978). Characterization of these antisera was carried out with high dilutions of the antisera in radioimmuno-

no assay models and later also in experiments with more concentrated dilutions, where each antiserum was preabsorbed with the other two peptides (100 µg of each peptide in a final antiserum dilution 1:100) without changes in the immunofluorescence staining. It cannot be excluded, however, that the antisera may bind to unknown peptides with similar amino acid sequences. It must also be pointed out that the peptides used for antibody production were originally isolated and sequenced from porcine or bovine tissues, and that the exact structure of the corresponding human or feline peptides is unknown. It therefore seems appropriate to use terms such as SP-like, ENK-like and VIP-like immunoreactivity or immunoreactive material.

#### Immunohistochemical procedure

Six adult cats of both sexes were used. Three of these were treated with local applications of vinblastine (Sigma) (Dahlström, 1971) on the serosal surface of the corpus, antral and pyloric regions of the stomach. Twenty-four hours later the stomachs were perfused with 2 000 ml of ice-cold 4% phosphate-buffered formaldehyde. The vinblastine treatment is known to raise peptide levels of the nerve cell bodies because of interrupted axonal transport (Dahlström, 1971; Lundberg et al., 1978). The three parts of the stomach were dissected out and immersion fixed for ~ hours in the same fixative and then rinsed in 5% sucrose in phosphate buffer at +4°C overnight.

The specimens were then processed for the indirect immunofluorescence technique according to Coons (1958). Briefly, 10 µm thick consecutive sections were incubated with antisera to met-ENK, VIP or SP (preabsorbed with BSA 50 mg/ml) when diluted 1:10, in a final dilution of 1:100–200 (containing 0.3% Triton X-100) for 24 hours at +4°C. The sections were then rinsed in phosphate-buffered saline (PBS) and incubated with FITC (fluorescein isothiocyanate)-conjugated sheep anti-rabbit antibodies (SBL, Stockholm, Sweden) diluted 1:4 for 30 min at 37°C. The sections were rinsed again in PBS then mounted in PBS-glycerine (1:3) and examined in a Zeiss fluorescence microscope equipped with an oil dark field condenser and a Schott BG-12 or alternatively a KP 500 excitation filter and a Zeiss 50 or LP 250 stop filter. Adjacent control sections were incubated with the antisera preabsorbed for 24 hours with an excess of respective peptide i.e. 100 µg met-ENK/ml antiserum diluted 1:160, 50 µg VIP or SP/ml antiserum diluted 1:100.

#### Collection of vagal nerves

Previous studies on neuropeptides in the axons of other species have indicated the necessity of a nerve crush (Lubinska, 1959) to raise the peptide levels to a level detectable with immunohistochemistry (Lundberg et al., 1979).

In 8 patients undergoing various kinds of thoracic or abdominal surgery 2 right thoracic main trunks, 2 anterior subdiaphragmatic trunks and 4 anterior nerves of Latarjet were collected. 30 min to 4 h before dissection the nerves were crush-operated with a silk suture compressing the nerve against a glass or metal rod for 5 sec and then released. The

segment 10 mm above and 5 mm below the crushed nerve was dissected and immersion fixed in ice-cold 4 % phosphate-buffered paraformaldehyde for 8 hours and rinsed in phosphate buffer containing 5 % sucrose over night. The nerves were then processed for the indirect immunofluorescence technique as described in (V).

## METHODOLOGICAL CONSIDERATIONS

The vast majority of previous *in vivo* studies of the pylorus region have been performed by means of pressure recordings or recordings of the electrical activity (Thomas & Wheldon, 1922, Brink et al 1965 Flaher & Cohen 1973 Mroz & Kelly 1977). Pressure recording systems have some disadvantages when gastrointestinal sphincters are investigated. It may be difficult to keep the pressure receptor within the sphincter region, which may also be difficult to exactly identify. Furthermore the recording device might interfere with sphincter motor activity. Recordings of electrical activity or contractility recorded by extramural strain gauge force transducers do not allow the study of overall motor function in such regions of the gastrointestinal tract. This may explain some of the differences between previous studies of the pyloric sphincter. In order to avoid some of these disadvantages and to be able to study the overall motor function of the pyloric region a flow recording method was used. Thus, the interference from a balloon or catheter within the sphincter region was avoided and even small variations in sphincter tone could be studied. However when a perfusion method is used several factors have to be taken into consideration. The perfusion pressure has to be kept at a constant level using a reservoir with a large area and a recirculation pump. The perfusion and drainage tube must have a capacity which is larger than the maximal flow through the sphincter and the tubes have to be placed close to the sphincter in order to avoid secondary effects from the adjacent antrum and duodenum. Since saline was used as perfusion fluid, interference due to viscosity could be excluded. The temperature of the perfusion fluid has been shown to be of great importance (Pahlén 1975). Therefore, the temperature of the perfusion fluid was kept constant at 38 °C.

As has been pointed out by Cruveilhier (1845) Wertheimer (1891) and Civalero (1979) the stomach has a segmental innervation and the gastric vagal nerves have a fairly constant course. The pyloric area is mainly innervated by the pyloric branches from the hepatoduodenal nerve of Latarjet and also by the anastomosing branch from the anterior nerve of Latarjet (Civalero 1979). Both nerves innervate the pylorus from the hepatoduodenal ligament cranial to the ligatures. Great care was taken to exclude the mesentery of the stomach and the hepatoduodenal ligament with its vagal nerve supply from the pylorus from nerve lesions imposed at dissection.

In the experiments with i.a. drug infusions a catheter was introduced via the splenic artery in a retrograde direction with the tip of the catheter placed at the junction with the celiac artery. On terminating the experiments an ink infusion through this catheter showed a distribution to the relevant region, that is the antrum, pylorus and proximal part of the duodenum in all animals.

## RESULTS

### Physiological experiments on vagal neurotransmission by classical receptors

#### Efferent vagal nerve stimulation (I)

Unilateral and bilateral efferent cervical vagal nerve stimulation with activation of *high* threshold fibres elicited a reduction or cessation of the transpyloric flow after about 20 sec, whereas a rapid (within 4 – 5 s) contraction was obtained in the stomach.

In 10 of 29 animals efferent vagal stimulation of the *high* threshold fibres resulted in a small but clear immediate initial increase of the transpyloric flow followed by a reduction of the flow. There was no difference in the pyloric or gastric response whether the splanchnic nerves were cut or left intact (Fig. 2 a).

Unilateral vagal stimulation activating the *low* threshold set of fibres, reduced the transsphincteric flow within 20 seconds in 2 of 5 cats. Bilateral stimulation activating the *low* threshold fibres, reduced the transsphincteric flow in only 1 of 4 animals.

#### Efferent vagal nerve stimulation during concomitant splanchnic nerve stimulation or NA infusion (I)

In order to investigate the influence of sympathetic tone on the pyloric motor response, the vagal nerves were stimulated when the sphincter tone was increased by a concomitant splanchnic nerve stimulation or local i.a. infusion of NA. The effect of vagal stimulation during simultaneous splanchnic nerve stimulation was studied in 5 experiments. Splanchnic nerve stimulation with supramaximal currents at 8 Hz elicited a contraction of the pylorus. Bilateral efferent vagal stimulation with activation of the *high* threshold fibres caused an *increased* transsphincteric flow in all animals.

In 2 animals the effect of vagal stimulation before and during NA infusion (0.01 – 0.07 mg/kg b.w./min i.a.) was studied. The infusion caused in these experiments a contraction of the sphincter. Efferent vagal stimulation during concomitant NA infusion caused an *increase* of the reduced flow in both animals studied.

#### Efferent vagal nerve stimulation after pretreatment with atropine, guanethidine and hexamethonium (II)

When 29 animals were pretreated with Atr (0.2 mg/kg i.v.) the nerve stimulation with *high* threshold parameters resulted in a gastric relaxation in all animals, whereas the pyloric motor response had a pronounced *biphasic* appearance in 24 animals consisting of an initial relaxation followed by a contraction some 60 sec later (Fig. 2 b).

In 5 animals the pyloric motor response changed into a pure *monophasic* relaxation. The vagally induced relaxation of the stomach and the pylorus was prompt, whereas the onset of the pyloric contraction had a significantly ( $p < 0.005$ ) longer latency after Atr than the pyloric contraction in the untreated animal.

In 9 animals Gua was combined (2 mg/kg i.v.) with Atr. In all of these animals the gastric relaxation and biphasic pyloric motor responses recorded at nerve stimulation were similar to those recorded from animals given Atr only.

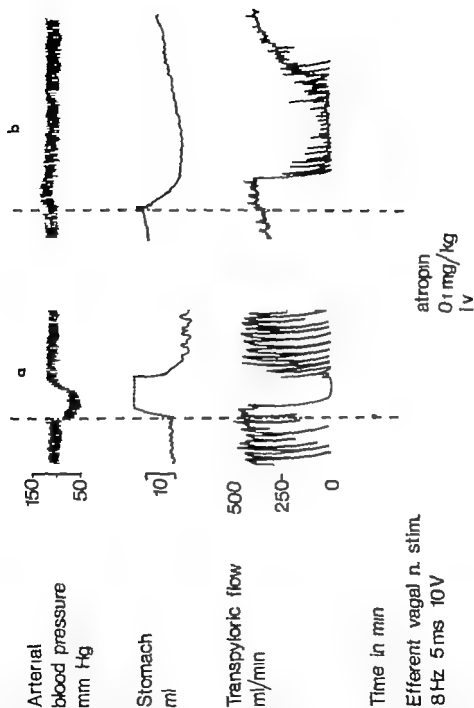


Fig. 2. The effect of efferent vagal nerve stimulation of the high threshold fibres on *a.* blood pressure, gastric and pyloric motility before and after the administration of atropine (0.1 mg/kg i.v.). Nerve stimulation in this animal resulted in a prompt gastric contraction and a late cessation of the transpyloric flow. However, after atropine gastric relaxation and a pronounced biphasic pyloric motor response was obtained with an initial increase and a delayed cessation of the flow. Note also the absence of blood pressure reaction after the drug pretreatment.

In 12 cats nerve stimulation was carried out after Hex (25 mg/kg i.v. followed by  $50 \pm 10$  mg/kg b.w./hr i.a.). Gastric motility was recorded in 10 of these animals. In 9 cats stimulation resulted in a gastric relaxation. In 11 cats there was a vagally induced pyloric relaxation, and in one cat there was both a gastric contraction and pyloric contraction.

When Atr was added to Hex in these 12 cats (or Hex was added to Atr in 4 other animals) the gastric motor response on nerve stimulation was completely blocked in all 16 animals. The pyloric relaxation was blocked in 14 animals and persisted in 2 animals.

### Afferent vagal nerve stimulation (II)

Afferent stimulation of the cervical vagal nerve with the contralateral nerve intact resulted in a reduction of the transpyloric flow and a concomitant gastric relaxation when the *high* threshold fibres were activated. Activation of the *low* threshold fibres resulted in a pyloric contraction in a few animals (Fig. 3). Reduction of the transsphincteric flow on afferent nerve stimulation followed after  $5 \pm 3$  s, whereas gastric relaxation was always prompt.

In 4 animals with a preganglionic denervation of the splanchnic nerves the cut proximal end of the vagus was afferently stimulated before and after division of the contralateral vagus. Vagal stimulation elicited a pyloric contraction and gastric relaxation in all 4 animals. These responses disappeared completely after transection of the contralateral vagal nerve. Efferent stimulation of either vagal nerve again induced a contraction of the pylorus.

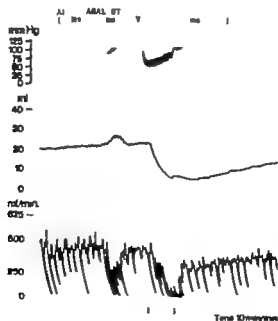


Fig. 3. The effect of afferent vagal nerve stimulation using low and then high threshold parameters on i.a. blood pressure, gastric and pyloric motility. Activation with either low or high threshold parameters elicited pyloric contraction, only the high threshold parameters elicited gastric relaxation.



Afferent vagal nerve stimulation after pretreatment with atropine, guanethidine and hexamethonium (II)

Afferent vagal nerve stimulation was given after pretreatment with Atr in 14 cats. The vagally induced contraction was blocked in 3 animals, and remained unaffected in 11 animals. In 5 animals Gua was added to the Atr pretreatment, a procedure which did *not* block the pyloric contraction in any of the animals.

In 10 animals nerve stimulation was carried out after Hex pretreatment. In 8 of these 10 animals the pyloric contraction was completely blocked, and in 2 animals it persisted.

### Immunohistochemistry of peptides in the vagus and the pylorus

#### Immunohistochemistry of the pylorus and stomach (III)

The two neuropeptides, ENK and VIP were both localized to numerous nerve fibres in the circular smooth muscle layer and the myenteric plexus of the feline pylorus. After pretreatment with vinblastine ENK and VIP immunoreactive nerve cell bodies could be demonstrated in the myenteric plexus of the pylorus. The most striking difference between the pylorus and the stomach was the presence of a dense network of nerve fibres with ENK- and VIP-like material running in parallel with the smooth muscle fibres of the circular muscle layer of the pyloric sphincter but only a few fibres were demonstrated within the same layer of the stomach. In vinblastine-treated animals the number of visible ENK and VIP nerve cell bodies of the myenteric plexus was greatly increased, especially in the pyloric region. The number of visible nerve cell bodies was much lower in the antrum - corpus. VIP immunoreactive nerve cell bodies were also observed in high numbers in the submucous plexus together with a dense network of VIP immunoreactive nerve fibres in the mucosal lamina propria of both the pylorus and the stomach. A dense network of ENK positive nerve terminals was also observed in the myenteric plexa of all the gastric regions investigated (see Table I).

#### Substance P, VIP and ENK-like immunoreactivity in the human vagus nerve (V)

Immunoreactive material of the three peptides SP, VIP and ENK were found in the axons of the vagal nerves at all levels studied as indicated by the appearance of fluorescent nerve fibres proximal to the ligation. The relative proportions of the peptides in the three branches investigated that is the thoracic main trunk, the anterior subdiaphragmatic trunk, and the anterior nerve of Latarjet were rather similar: very high numbers of SP-fibres, somewhat fewer ENK fibres and a few VIP axons in each nerve segment. The accumulation of immunoreactive material above the ligation was seen after 1 hour and increased with time indicating a rapid anterograde axonal transport of the peptides. Thin ENK axons could even be demonstrated far away from the crush on both sides within the nerve of Latarjet. Control sections were negative as described in (V).

*Table 1 Localization and estimation of the abundance of VIP and ENK-immunoreactive material within nerve fibers and nerve cell bodies in the gastric and pyloric wall of the cat.*

	PYLORUS		STOMACH	
	ENK	VIP	ENK	VIP
LAMINA PROPRIA MUCOSAE	—	nerves ++	—	nerves ++
SUBMUCOUS PLEXUS	—	ganglion cells ++	—	ganglion cells ++
CIRCULAR MUSCLE LAYER	nerves ++	nerves ++	nerves (+)	nerves +
MYENTERIC PLEXUS	nerves ++ ganglion cells ++	nerves ++ ganglion cells + (+)	nerves ++ ganglion cells (+)	nerves + ganglion cells (+)
LONGITUDINAL MUSCLE LAYER	nerves (+)	nerves (+)	nerves (+)	nerves (+)

nerves = axons and nerve terminals

(+) few

+ moderate number or medium

++ high number or dense

## Physiological experiments on vagal neurotransmission by non-classical receptors

### Vagal nerve stimulation before and after naloxone and/or atropine (III)

During infusion of Nal there was an increasing delay of the pyloric contraction whereas the gastric contraction remained unchanged at efferent vagal nerve stimulation using *high* threshold parameters. A final blockade of the pyloric response was obtained in all cats examined (6/6). The vagally mediated cholinergic gastric contraction seemed to be unaffected during i.s. infusion of Nal as indicated by the response to ACh injection. When Atr was given to the animals, *high* threshold vagal stimulation still elicited a pyloric contraction whereas the gastric response had changed into a relaxation. During Nal infusion an increasing blockade of the pyloric contraction at vagal nerve stimulation was seen in all 9 cats. A total blockade was observed in 6/9 cats at a total dose of 2.5 mg (6.9  $\mu$ mol) of the drug. In the remaining 3 animals, there was a dose dependent increasing delay of the pyloric motor response and the contraction was completely blocked at a total dose of 8 mg (16.6  $\mu$ mol). The Atr resistant gastric relaxation was also affected but to a lesser extent (Fig. 4).

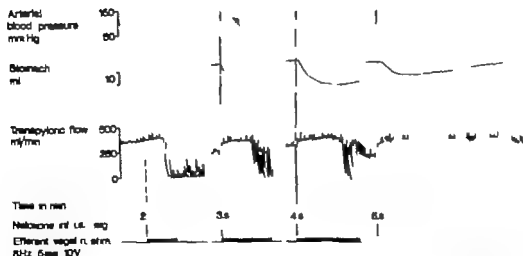


Fig. 4. Recordings of i.s. blood pressure, gastric motility and transpyloric flow in an atropine pretreated (0.1 mg/kg i.s.) animal during electrical vagal nerve stimulation in efferent direction, using *high* threshold parameters and i.s. infusions of naloxone as indicated in the figure (from III with permission of Gastroenterology).

### Intraarterial injections of morphine met and leu-enkephalin and naloxone (III)

The I.A. injection of Mo (8 cats) caused a delayed cessation (within 1 – 3 min) of the transpyloric flow (duration 4 – 5 min) in all cats and induced a slow increase in the gastric tone which lasted for 20 – 30 min in 7 of 8 cats induced with a dose of 0.5 mg (1.5  $\mu$ mol) or more. The lowest tested dose 0.1 mg (0.3  $\mu$ mol) resulted in a 50 – 70 % reduction of the transpyloric flow (2 cats) which lasted for a few seconds only. The administration of equimolar bolus doses of Nal blocked the Mo effects on both the pylorus and the stomach. The infusion of met or leu ENK (0.08 – 116  $\mu$ mol) (5 and 4 cats respectively) elicited an immediate contraction of the pylorus of shorter duration (up to 3 min) than that obtained with Mo. This effect on the transpyloric flow was almost abolished by pretreatment with Nal infusions. A prompt gastric relaxation (in contrast to the slow contraction obtained with Mo) was obtained which was also blocked by Nal. Pretreatment with Atr or Hex did not affect the responses elicited by I.A. injection of ENK. The specificity of the Nal blockade was tested by the injection of ACh in 5 cats. ACh induced a ceased transpyloric flow and a gastric contraction before and after the administration of Nal in all animals (Fig. 5).

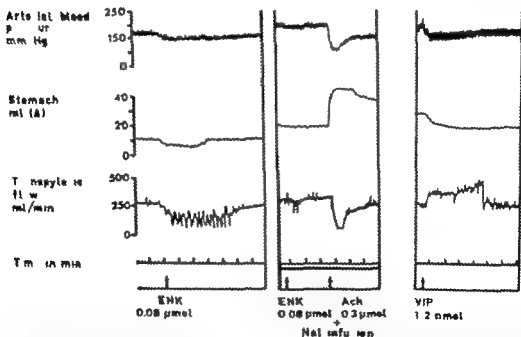


Fig. 5 Recordings of I.A. blood pressure, gastric motility and transpyloric flow in the cat during I.A. bolus injections of ENK (0.08  $\mu$ mol) and VIP (1.2 nmol) respectively. ENK caused a pyloric contraction and gastric relaxation, both effectively blocked by naloxone infusion (total dose 6.9 – 16.6  $\mu$ mol). The cholinergic receptors seemed intact. VIP caused both a pyloric and gastric relaxation.

### Intraarterial injections of VIP (IV)

The intraarterial injection of VIP in 7 cats induced a prompt gastric relaxation and an immediate increase in the transpyloric flow at all doses given (0.03 – 1.3 nmol) but only in 50 % of the experiments with the lowest dose (0.03 nmol). The VIP effects could not be blocked with Atr or Hex at the doses previously used (II) (Fig. 5).

## DISCUSSION

### Physiological experiments on vagal neurotransmission by classical receptors (I–II)

In the present series of experiments *efferent* cervical vagal nerve stimulation with physiological frequencies and supramaximal currents caused a monophasic contraction of the pylorus with a latency of  $23 \pm 17$  s in two thirds of the animals. In the remaining animals, an initial relaxation of the pylorus with an increased transsphincteric flow with a latency of  $5 \pm 3$  s followed by a contractile phase of the sphincter was recorded. This may indicate the presence of both excitatory and inhibitory vagal nerve fibres to the pylorus. To further investigate the vagal control of the pyloric sphincter region the vagal nerves were stimulated during a concomitant increase in sphincter tone since the type of intestinal response to vagal stimulation is considered to be determined by the tonus (Garry 1957 van Harn, 1963). Thus low tonus of the intestine may favour an excitatory response and high tonus an inhibitory response. When pyloric tonus was increased with NA infusion or splanchnic nerve stimulation vagal activation caused an increased flow i.e. an inhibitory response in both these series of experiments. This supports the assumption that the vagal nerves convey both excitatory and inhibitory fibres, not only to the stomach but to the pylorus as well as earlier suggested by Langley (1898) Anuras et al. (1974) Martinson (1965 a, b) Mir et al. 1979).

*Afferent* cervical vagal stimulation with physiological frequencies and supramaximal currents with the contralateral vagal nerve left intact resulted in a contraction of the pylorus within 5 sec, as well as prompt gastric relaxation. Both responses disappeared when the contralateral cervical vagal nerve was cut indicating the presence of a vago-vagal excitatory reflex activation of the pylorus as well as a similar inhibition of the stomach.

It has previously been shown that vagal nerves contain at least two groups of *efferent* fibres which control gastric motor activity (Martinson and Muren, 1963). The excitatory fibres were selectively activated when the *low* threshold parameters were used. If the duration of the stimulatory pulses was increased to supramaximal currents, *high* threshold vagal relaxatory fibres were activated as well. Activation of the *low* threshold fibres induced an inconsistent excitatory motor response of the pylorus in the present series of experiments, but relaxatory responses were not seen. Activation of the *high* threshold fibres caused pyloric motor responses as described above.

In order to study the transmission mechanisms involved in the vagally induced excita-

tory and inhibitory pyloric motor responses high threshold parameters were used in both efferent and afferent stimulations after the administration of classical cholinergic adrenergic and ganglionic blocking agents.

*Efferent vagal stimulation* after the administration of *atropine* resulted in a gastric relaxation in all animals thus confirming previous findings (Martinson et al., 1965 a). The pyloric motor response was biphasic in the majority of the animals, an immediate relaxation followed by a delayed contraction with a significantly longer latency than seen before Atr was given. In a few animals a monophasic relaxation of the pylorus was obtained. Motor responses on *efferent vagal nerve stimulation* after Atr were unaffected in the vast majority of the experiments. However in a few animals a blockade of the pyloric contraction was seen. Atr in the doses given in the present series of experiments (e.g. 0.1 – 0.2 mg/kg) is known to completely block the vagally induced excitatory motor effects on both the stomach, the small intestine the ileo-caecal sphincter and the colon (Martinson 1965 Kewenter 1965 Hultén, 1969 Pahlén, 1975). Furthermore the low dose of Atr used in the present series of experiments is considered to have a specific anti cholinergic action on muscarinic receptors (Innes & Nicholson, 1975). However such a blockade did not effectively prevent a pyloric contraction except in a few animals. An additional effect of atropine cannot therefore be completely excluded.

When *guanethidine* was given after Atr the gastric as well as the pyloric motor responses at *efferent* and *afferent vagal stimulation* were similar before and after administration of Gua. Thus, an adrenergic transmission mechanism can be excluded. *Hexamethonium* blocked the gastric as well as pyloric contraction in all but one animal on *efferent stimulation* and in all but two cats on *afferent stimulation*, indicating a ganglionic transmission (Voile & Koelle, 1975). However the relaxatory motor responses in both the stomach and the pylorus remained unchanged.

When a combination of Atr and Hex was given, the gastric relaxation was blocked in all animals and the pyloric relaxation in 14/16 animals on efferent cervical vagal nerve stimulation. The total blockade of the gastric relaxation in these experiments is in contrast to the partial blockade described by Martinson et al. (1965 a). However it should be noted that the dose of Hex used here was different and that a constant i.a. infusion of the drug was given throughout the experiments.

*In conclusion*, Hex alone was effective in blocking the vagally elicited excitatory motor response of both the pylorus and the stomach indicating a ganglionic transmission step. Furthermore, the gastric excitatory responses seem to be mediated by cholinergic transmission, whereas the pyloric excitatory responses do not seem to be mediated by classical muscarinic receptors. However the non-cholinergic, non-adrenergic relaxatory responses of the pylorus and the stomach required the addition of Atr to Hex to be blocked (Fig. 6).

The results obtained after the administration of cholinergic and adrenergic ganglionic blocking agents on the vagally induced pyloric motor response suggest one or more of the following possibilities of neural transmission. Thus, both nicotinic and muscarinic receptors seem to be involved in the synaptic transmission either at the ganglionic level or the effector cell level, or both, since a combination of Atr and Hex had to be given to block

the relaxatory pyloric motor response. A ganglionic transmission has previously been suggested in the vagal inhibitory control of the lower oesophageal sphincter (Goyal & Rattan 1975) and also for the non-cholinergic non-adrenergic receptive relaxation of the stomach (Martinson, 1965 a, b). Since some of the vagal nerve fibres are postganglionic (Schofield et al. 1968) the occasional persistent vagally induced contractions after Hox on of efferent stimulation might also be explained by the activation of such fibres. An antidromic stimulation of *efferent* sensory vagal fibres cannot be completely excluded since such an activation may cause a release of products that relax sphincter muscles (Burnstock, 1972).

An interesting finding in the present experiments was that the pyloric contraction elicited by *efferent* vagal nerve stimulation was seen after a delay of about 20 sec. This is in contrast to the almost immediate contraction seen in the stomach, small intestine ileocecal valve and colon induced by efferent vagal stimulation (Martinson 1965 a, b; Lewerter 1965; Hultén, 1969; Pahlén 1975). This rather long latency may indicate the activation of composite neurohumoral mechanisms such as the release of gastrointestinal hormones, or simply reflect that the innervation of sphincter regions is more complex than that of the gut itself (Goyal & Rattan 1975). A procedure such as *efferent* electrical stimulation of the vagi activates not only cholinergic and adrenergic fibres, but probably also peptidergic fibres (Uvnäs-Wallensten, 1978; Lundberg et al. 1978 a, b) present in this nerve. Furthermore it is known that monoamines (Ahlmán, 1976) as well as peptidergic hormones (Uvnäs-Wallensten et al. 1976) with potent actions on gut motility are released by such stimulations. The delayed pyloric contraction on vagal *efferent* stimulation may therefore be due to a release of such substances or both into the gut lumen and/or into the blood stream. However it has been shown that the relaxation as well as the "off motor response" of the canine pylorus following vagal stimulation seem to be nerve mediated, since tetrodotoxin blocked these responses (Telford et al. 1979).

In order to find what possible neurotransmitters were responsible for the non-cholinergic non-adrenergic motor effects found in the present studies the vagus nerve, the gastroduodenal junction and the stomach were investigated using immunohistochemical techniques for the presence of several neuropeptides.

### Immunohistochemistry of peptides in the vagus and the pylorus of the cat (III)

Immunohistochemical studies in the cat revealed that the pyloric region had much denser networks of nerve fibres with ENK-immunoreactive material in the circular muscle layer than the stomach. In contrast to a previous study (Linnoila et al., 1978) ENK-positive nerve cell bodies were found in the myenteric plexuses with a much higher concentration in the pyloric region than in the antrum-corpus part of the stomach. In the myenteric plexus dense networks of ENK-positive nerve-terminal-like structures were observed at all levels of the stomach in agreement with previous studies (Elde et al. 1976; Polak et al., 1977).

The immunohistochemical studies demonstrated numerous VIP immunoreactive cell bodies in both intramural plexuses of the pylorus, with the highest number in the submucous plexus. VIP-immunoreactive nerve fibres were seen in the myenteric plexus, circular muscular layer and lamina propria mucosae of the pylorus.

The number of VIP immunoreactive cell bodies in the myenteric plexus was less in the stomach than in the pylorus, but approximately the same amount were found in the submucous plexuses of the pylorus and stomach. The circular muscular layer of the antrum-corpus contained a medium dense network of VIP-immunoreactive nerve fibres while in the lamina propria a dense network was seen and only scattered nerve fibres in the longitudinal muscle layer.

VIP-containing nerve fibres have previously been demonstrated in other sphincter regions than the pylorus, such as the lower oesophageal sphincter and the sphincter of Oddi (Alumets et al., 1978), gastrointestinal and genito-urinary tract (Alumets et al., 1979; Larson et al. 1976). The numerous VIP-immunoreactive fibres of the lamina propria both in the stomach and the pylorus may be of importance for secretion and mucosal blood flow as suggested by Fahrenkrug et al. (1978 b) and Eklund et al. (1979).

### Physiological experiments on vagal neurotransmission by non-classical receptors (III-IV)

Since immunohistochemical studies (III) revealed a rich ENK-ergic and VIP-ergic innervation of the pyloric region II was of interest to experimentally study if ENK and VIP are involved in the vagal neurotransmission to the pylorus.

When the cervical vagal nerves were stimulated in an *efferent* direction with supramaximal currents and physiological frequencies during I.a. infusion with the opiate receptor antagonist Nal the pyloric contraction was inhibited in a dose dependent manner. These experiments were repeated after the administration of 4tr to investigate the Nal-effect on gastric relaxation. It was then found that the vagally induced gastric relaxation was also influenced by Nal-infusion, but to a lesser extent than the pyloric contraction, and the gastric relaxation was never completely blocked. This suggests that opiate receptors are involved in these vagally mediated motor responses, although the receptors in the pylorus and stomach may be slightly different. In order to further investigate this hypothesis the natural agonists of opiate receptors, enkephalines (leu- and met-ENK), were injected I.a. to the pyloric and the lower stomach regions since if an ENK-ergic pathway were involved in the vagally induced non-cholinergic, non-adrenergic motor response these substances should mimic the vagally elicited responses. This was also found to be the case since leu- and met-ENK I.a. elicited a pyloric contraction and a gastric relaxation. These responses were completely abolished by Nal, an effect which seemed to be selective for ENK since the Ach induced motor responses were unaffected by Nal-infusion. Intrarterial infusion of other opiate receptor agonists such as Mo and ENK-analogs elicited similar responses as met- and leu-ENK in the pylorus, but instead caused a slow increase of the gastric tone. These effects could be blocked by equimolar bolus doses of Nal. On a molar basis ENK



was about 20 times more potent than Mo although the Mo-effect was more longlasting. In most other systems the opposite is observed where Mo is found more potent (Snyder & Childers, 1979; Ambinder & Schuster 1979). There is, however, evidence for multiple opiate receptors. A certain subclass of receptors, delta receptors, which have a high affinity for ENK and low affinity for opiate alkaloids including the opiate antagonist Nal (Lord et al., 1977; Terenius 1977; Kosterlitz & Leslie 1978) has been suggested. Since ENKs are very unstable in plasma, it has so far not been possible to establish if ENK is released to the venous blood from the pylorus on efferent vagal nerve stimulation (Dupont 1977; Sullivan et al. 1978).

The rapid enzymatic degradation of ENKs in plasma suggests that the humoral transport of ENK to a distant site of action is less likely (Teaschmacher et al., 1978). However, the results of the present study indicate that ENK-ergic nerve fibres are involved in the control of pyloric contractility. Although several of the present findings strongly indicate that ENK is a neurotransmitter in the gastrointestinal tract more experimental work has to be done before this can be fully established.

Besides the rich ENK-ergic innervation of the pyloric region the immunohistochemical studies also demonstrated VIP-containing nerve terminals and VIP-immunoreactive nerve cell bodies in the pyloric region as well as VIP-like immunoreactivity within vagal axons. Therefore, it was also of interest to study the effect of VIP on the pyloric and gastric motor function (IV).

Close i.a. injection of VIP to the pylorus and stomach caused a prompt increase of the transpyloric flow and a relaxation of the stomach. These effects could not be blocked by neither Atr nor Hex. The VIP-induced gastric relaxation which was similar to the receptive relaxation was of a rather long duration (10 – 15 min) whereas the pyloric relaxation was much shorter in duration (4 – 5 min). These studies confirm that VIP seems to have a general relaxatory effect on the gastrointestinal tract (Morgan et al., 1978) but that the duration of the response may vary between different parts of the gastrointestinal tract. A similar prolonged inhibitory response of the stomach to VIP injection has previously been shown by Eklund et al. (1979).

VIP has previously been shown to relax the cardia sphincter of the opossum (Rattan, 1977) and vagal nerve stimulation has also been shown to significantly increase the venous plasma VIP concentration (Fahrenkrug et al., 1978 b). These authors have therefore suggested that VIP is a neurotransmitter in the gastrointestinal tract (Fahrenkrug, 1979) probably mediating the non-cholinergic non-adrenergic receptive relaxation. It is also possible that the vagally induced pyloric relaxation is mediated via a similar mechanism.

*In conclusion:* It was found that 1) the i.a. injection of met- and leu-ENK caused a pyloric contraction and a gastric relaxation. The effects were possible to block or reduce with Nal in a dose dependent manner. 2) ENK-amide, on the other hand, caused a contraction of both the stomach and the pylorus, and these effects could also be antagonized by Nal. 3) The pyloric contraction elicited on efferent cervical vagal stimulation was inhibited by Nal in a dose dependent manner. 4) The second peptide, VIP, with a similar morphological distribution as ENK, caused a pyloric and gastric relaxation at i.a. injec-

tion (Fig. 5) The results of the immunofluorescence and physiological studies indicate that ENK and VIP may serve as neurotransmitters in the pylorus and stomach. (Fig. 6)

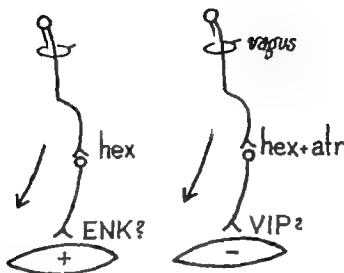


Fig. 6 Hypothetical interpretation of the present immunohistochemical studies of the pylorus in combination with the findings in experiments on the transmission mechanism of the vagal control of the f. l. pylorus.

### Immunohistochemistry of human vagal nerve (V)

Immunohistochemical studies were performed on human vagal nerves (V) in order to investigate whether peptidergic neurotransmission is involved in the motor control of the pylorus and stomach *in vivo*. A high number of SP fibres, medium numbers of ENK fibres, and a few VIP fibres were found in these vagal nerves examined, that is the thoracic nerve trunk, anterior subdiaphragmatic vagal nerve and the nerve of Latarjet (V). Thus, the vagal nerve, both in man and in cat, was demonstrated to be a heterogeneous nerve containing several different peptide systems in addition to cholinergic and adrenergic systems. (Muryohayashi 1968, Burnstock 1972, Lundberg et al. 1976 and V). The present study indicates the presence of three peptides, SP, VIP and ENK, in the axons of the human vagus as revealed by immunohistochemical studies of nerves ligated *in vivo* and later extirpated during surgery. Since the peptides accumulate centrally to the ligation a peripherally directed axonal transport seems probable.

was about 20 times more potent than Mo although the Mo-effect was more longlasting. In most other systems the opposite is observed, where Mo is found more potent (Snyder & Childers, 1979; Ambinder & Schuster 1979). There is, however, evidence for multiple opiate receptors. A certain subclass of receptors, delta receptors, which have a high affinity for ENK and low affinity for opiate alkaloids including the opiate antagonist Nal (Lord et al., 1977; Terenius, 1977; Kosterlitz & Leslie 1978) has been suggested. Since ENKs are very unstable in plasma, it has so far not been possible to establish if ENK is released to the venous blood from the pylorus on efferent vagal nerve stimulation (Dupont 1977; Sullivan et al., 1978).

The rapid enzymatic degradation of ENKs in plasma suggests that the humoral transport of ENK to a distant site of action is less likely (Teschemacher et al. 1978). However, the results of the present study indicate that ENK-ergic nerve fibres are involved in the control of pyloric contractility. Although several of the present findings strongly indicate that ENK is a neurotransmitter in the gastrointestinal tract, more experimental work has to be done before this can be fully established.

Besides the rich ENK-ergic innervation of the pyloric region, the immunohistochemical studies also demonstrated VIP-containing nerve terminals and VIP-immunoreactive nerve cell bodies in the pyloric region, as well as VIP-like immunoreactivity within vagal axons. Therefore, it was also of interest to study the effect of VIP on the pyloric and gastric motor function (IV).

Close i.a. injection of VIP to the pylorus and stomach caused a prompt increase of the transpyloric flow and a relaxation of the stomach. These effects could not be blocked by neither Atr nor Hex. The VIP induced gastric relaxation, which was similar to the receptive relaxation, was of a rather long duration (10–15 min), whereas the pyloric relaxation was much shorter in duration (4–5 min). These studies confirm that VIP seems to have a general relaxatory effect on the gastrointestinal tract (Morgan et al., 1978) but that the duration of the response may vary between different parts of the gastrointestinal tract. A similar prolonged inhibitory response of the stomach to VIP injection has previously been shown by Eklund et al. (1979).

VIP has previously been shown to relax the cardia sphincter of the opossum (Rattan, 1977) and vagal nerve stimulation has also been shown to significantly increase the venous plasma VIP concentration (Fahrenkrug et al., 1978 b). These authors have therefore suggested that VIP is a neurotransmitter in the gastrointestinal tract (Fahrenkrug, 1979) probably mediating the non-cholinergic, non-adrenergic receptive relaxation. It is also possible that the vagally induced pyloric relaxation is mediated via a similar mechanism.

*In conclusion*, it was found that 1) the i.a. injection of met- and leu-ENK caused a pyloric contraction and a gastric relaxation. The effects were possible to block or reduce with Nal in a dose dependent manner. 2) ENK-amide, on the other hand, caused a contraction of both the stomach and the pylorus, and these effects could also be antagonized by Nal. 3) The pyloric contraction, elicited on efferent cervical vagal stimulation, was inhibited by Nal in a dose dependent manner. 4) The second peptide, VIP, with a similar morphological distribution as ENK, caused a pyloric and gastric relaxation at i.a. injection.

seems suitable for such an action. The physiological stimuli for this reflex are not established but an increased discharge of vagal afferent fibres has been demonstrated on stimulation of either chemo- or mechano-receptors of the intestine, stomach and oesophagus (Andrew, 1957; Iggo, 1957; Harper et al., 1959; Paintal, 1954).

The integrated gastric response to vagal stimulation seems meaningful for the digestion of food. It induces a receptive relaxation and a concomitant closure of the pylorus. The low threshold vagal fibres facilitate movements of the stomach, which are necessary to mix the food with acid and pepsinogen, also under vagal influence (Martinson, 1965 b). All these activities require an increased blood supply regulated by vagal nerves. The non-cholinergic non-adrenergic transmission involved in some of these mechanisms is still unclear but it is interesting to note that an infusion of ENK into a gastric artery has been shown to increase acid and pepsinogen secretion and the mucosal blood flow (Konturek et al., 1978) and to further cause a pyloric contraction and gastric relaxation, as shown in the present series of experiments.

## GENERAL CONCLUSIONS

The following conclusions were drawn on the basis of the observations made

1. The vagal nerves in cat contain both excitatory and inhibitory fibres to the pylorus. Both effects seem to be mediated via non-cholinergic, non-adrenergic mechanisms in contrast to the cholinergic gastric contraction. A vago-vagal excitatory reflex to the pylorus was elicited by afferent vagal nerve stimulation together with a vago-vagal gastric relaxation.
2. Within the feline pylorus numerous nerve fibres with ENK-like immunoreactivity both in the circular muscle layer and the myenteric plexus were demonstrated. Many cell-bodies with ENK-like material were demonstrated in the myenteric plexus. The pyloric region was far more densely innervated with ENK-positive nerve fibres than the other parts of the stomach. Within the circular muscle layer VIP-immunoreactive fibres had a distribution very similar to the ENK-positive nerves.
3. Local intramural infusion of the opiate antagonist naloxone blocked the pyloric contraction obtained on efferent vagal nerve stimulation in a dose dependent manner. VIP or ENK injected i.a. resulted in a gastric relaxation while the two peptides had antagonistic effects on the pyloric motility. VIP inducing relaxation and ENK contraction. These results suggest a vagal control of the feline pylorus and stomach which is mediated by these peptides.
4. The presence of axons in the human vagus with SP, ENK and VIP-like immunoreactivity was demonstrated.

## ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to

My friends and teachers Docent Jan Kowenter who introduced me into the field of gastrointestinal physiology. He has followed these studies with enthusiastic and constructive criticism. Docent Håkan Ahlman who introduced me into the field of fluorescence histochemistry. He has guided me throughout this investigation with never failing interest and encouragement. I am deeply grateful to him for this. Docent Annica Dahlström whose interest and support have greatly contributed to the completion of this work. Med. kand Jan Lundberg who has made valuable and major contributions to this study. I am also indebted to professors Yngve Edlund and Lars-Erik Gelin for their support given to this study.

For most skilful technical assistance I want to thank Mrs. Harriet Andersson and Miss Ylva Larsson.

Miss Anita Pettersson, Miss Karin Linnér and Miss Inga-Lill Bye for expert secretarial work.

Dr. Andrew Heath for revising the English text.

My family for patience and understanding.

This study was supported by the Swedish Medical Research Council (grants No 17X-5220, 04X-2207, 04P-4173, 04X-2887) by the Medical Faculty, University of Göteborg, Göteborgs Läkaresällskap, Magnus Bergwalls stiftelse and Harald & Greta Jeansson's stiftelse.

## REFERENCES

- AGOSTONI, E., CHINNOCK, J.E. De BURGH DALY M & MURRAY J.G. 1957 Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. *J Physiol* 135 182-205
- AHLMAN H. 1976 Fluorescence histochemical studies on serotonin in the small intestine and the influence of vagal nerve stimulation. *Acta Physiol Scand Suppl* 437
- ALUMETS J., HÅKANSSON R. & SUNDLER, F. 1978 VIP innervation of sphincters. *Scand J Gastroenterol* 13 Suppl 49 75
- ALUMETS J. FAHRENKRUG J. HÅKANSSON R., SCHAFFALITZKY DE MUCKA DELL, O. SUNDLER F. & UDDMAN R. 1979 A rich VIP nerve supply is characteristic of sphincters. *Nature* 280 155-156
- AMBINDER R.F. & SCHUSTER, M.M. 1979 Endorphins: New gut peptides with a familiar face. *Gastroenterology* 77 1132-1140
- ANDREW B.L. 1957 Activity in afferent nerve fibres from the cervical esophagus. *J Physiol (Lond)* 135 54-55
- ANURAS S., COOKE, A.R. & CHRISTENSEN J. 1974 An inhibitory innervation at the gastroduodenal junction. *J Clin Invest* 54 529-535
- BELDING H.H. & KERNOHAN J.W. 1953 A morphological study of the myenteric plexus and musculature of the pylorus with special reference to the changes in hypertrophic pyloric stenosis. *Surg Gynec Obstet* 97 322-334
- BERGER T. 1969 Studies on the gastric emptying mechanism. *Acta Chir Scand Suppl* 404
- BRINK, B.M. SCHLEGEL, J.F. & CODE C.F. 1965 The pressure profile of the gastroduodenal junctional zone in dogs. *Gut* 6 163-171
- BURHOL, P.G. WALDUM H.L. JORDE, R. & LYGREN L. 1979 The effect of a test meal on plasma vasoactive intestinal polypeptide (VIP), gastric inhibitory polypeptide (GIP) and secretin in man. *Scand J Gastroent* 14 (8) 939-943
- BURNSTOCK, G. 1972. Parasympathetic nerves. *Pharmacol Rev* 24 509-581
- CANNON W.B. 1906-1907 The motor activities of the stomach and small intestine after splanchnic and vagus section. *Amer J Physiol* XVII 429-442.
- CARLSON H.C. CODE, C.F. & NELSON R.A. 1966. Motor action of the canine gastroduodenal junction. *Amer J Digest Dis* 11 155-172
- CTVALERO L.A. 1979 Selective proximal vagotomy in duodenal ulcer. *Acta Chir Scand Suppl* 491
- COHEN S. HARRIS L.D. & LEVITAN R. 1968 Manometric characteristics of the human ileocecal junctional zone. *Gastroenterology* 54 77-75
- CODE, C.F. & CARLSON H.C. 1968 Motor activity of the stomach. In *Handbook of Physiology* (ed C.F. Code) pp 1903-1916. Williams & Wilkins Baltimore
- COONS, A.H. 1958 Fluorescence antibody methods. In *General cytochemical methods* (ed J.F. Damelli) New York Academic Press Inc pp 399-422.
- CRUVEILLIER, J. 1845 *Traité d'anatomie descriptive* Paris.

- CUNNINGHAM D.J. 1906 The varying form of the stomach in man and the anthropoid ape. *Trans Roy Soc, Edinburgh* 45 9-47
- DAHLSTRÖM A. 1971 Effects of vinblastine and colchicine on monoamine containing neurons of the rat with special regard to the oxoplasmic transport of amine granules. *Acta Neuropath (Berl) Suppl V* 226-237
- DIDIO L.J.A. & ANDERSSON M.C. 1968 The "sphincters" of the digestive system. *Williams & Wilkins* 95-118
- DUPLESSIS D.J. 1965 Pathogenesis of gastric ulceration. *Lancet* I 947-978
- DUPONT A., CUSAN L., GARON M., ALVARADO-URBINA, G. & LABRIE F. 1977 Extremely rapid degradation of methionine-enkephalin by various rat tissues in vivo and in vitro. *Life Sciences* 21 907-914
- EDWARDS D.A.W. & ROWLANDS E.N. 1968 Physiology of the gastroduodenal junction. In *Handbook of Physiology* (ed. C.F. Code) sect. 6 vol 7 p 1985. *Williams & Wilkins, Baltimore*
- EKLUND S., JODAL, M., LUNDGREN O. & SJÖQUIST A. 1979 Effects of vasoactive intestinal polypeptide on blood flow, motility and fluid transport in the gastrointestinal tract of the cat. *Acta Physiol Scand* 105 461-468
- ELDE, R., HÖKFELT T., JOHANSSON O. & TERENIUS L. 1976 Immunohistochemical studies using antibodies to leucin-enkephalin. Initial observations on the nervous system of the rat. *Neuroscience* 1 349-351
- ELLIOT T.R. 1904 On the innervation of the ileocolic sphincter. *J Physiol* XXXI 157-168
- FAHRENKRUG J. & SCHAFFALITZKY DE MUCKADELL, O.B. 1977 Radioimmunoassay of vasoactive intestinal polypeptide (VIP) in plasma. *J Lab Clin Med* 89 1379-1388.
- FAHRENKRUG J., GALBO H., HOLST J.J. & SCHAFFALITZKY DE MUCKADELL, O.B. 1978 a. Influence of the autonomic nervous system on the release of vasoactive intestinal polypeptide from the porcine gastrointestinal tract. *J Physiol (Lond)* 280 405-422
- FAHRENKRUG J., HAGLUND U., JODAL, M., LUNDGREN O., OLBE L. & SCHAFFALITZKY DE MUCKADELL, O.B. 1978 b. Nervous release of vasoactive intestinal polypeptide in the gastrointestinal tracts of cats. Possible functional implications. *J Physiol (Lond)* 284 291-305
- FAHRENKRUG J. 1979 Vasoactive intestinal polypeptide: Measurement, distribution and putative neurotransmitter function. *Digestion* 19 149-169
- FISHER R. & COHEN S. 1973 Physiological characteristics of the human pyloric sphincter. *Gastroenterology* 64 67-75
- FREDRICKSON R.C.A. 1977 Enkephalin pentapeptides - A review of current evidence for a physiological role in vertebrate neurotransmission. *Life Sci* 21 23-42.
- GARRY R.C. 1957 Innervation of abdominal viscera. *Brit Med Bull* 13 202-206.
- GASKELL, W.H. 1916 The involuntary system. Longmans, Green & Co. London 178 pages.

- GOYAL, R.K. & RATTAN S. 1975 Nature of the vagal inhibitory innervation to the lower esophageal sphincter. *J Clin Invest* 55 1119-1126
- HARN G.L. v 1963 Responses of muscles of cat small intestine to autonomic nerve stimulation. *Amer J Physiol* 204 352-358
- HARPER, A.A. KIDD C. & SCRATCHERD T. 1959 Vago-vagal reflex effects on gastric and pancreatic secretion and gastrointestinal motility. *J Physiol (Lond)* 148 417-436
- HORTON B T. 1928 Pyloric musculature with special reference to pyloric block. *Amer J Anat* 41 197-226
- HULTÉN L. 1969 Extrinsic nervous control of colonic motility and blood flow. *Acta Physiol Scand Suppl* 335
- HOLLT V & WÜSTER, M. 1978 The opiate receptors. In: *Developments in opiate research* (ed. A. Herz) chap. 1. Marcel Dekker, New York.
- IGGO A. 1957 Gastric mucosal chemoreceptors with vagal afferent fibres in the cat. *Quart J Exp Physiol* 42 398-409
- INNES I.R. & NICKERSSON M. 1975 Atropine, scopolamine and related antimuscarinic drug. In: *The pharmacological basis of therapeutics* (ed. L.S. Goodman & A. Gilman) pp. 514-532. MacMillan Publ Co Inc, New York.
- KATZ, A. & WINKLER, F. 1902. *Beitr Exp Path aus Lab von Basch*, Quoted from Hermann's Jahresberichte 1903
- KEWENTER, J. 1965 The vagal control of the jejunum and duodenum motility and blood flow. *Acta Physiol Scand Suppl* 251
- KOCK, N G. 1959 An experimental analysis of mechanisms engaged in reflex inhibition of intestinal motility. *Acta Physiol Scand* 47 Suppl 164
- KONTUREK, S. 1978 Endogenous opiates and the digestive system. *Scand J Gastroenterol* 13 257-261
- KOSTERLITZ, H.W. & LESLIE, F.M. 1978 Comparison of the receptor binding characteristics of opiate agonists interacting with  $\mu$ - or  $\kappa$ -receptors. *Brit J Pharmacol* 64 607-614
- LANGLEY J N. 1898-99 On inhibitory fibres in the vagus for the end of the esophagus and the stomach. *J Physiol* XXIII. 407-414
- LARSSON L.-L. FAHRENKRUG J. SCHAFFALITZKY DE MUCKADELL, O.B. SUNDLER, F., HÅKANSSON R. & REHFELD J R. 1976. Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. *Proc Nat Acad Sci, USA* Wash 73(9): 3197-3200
- LINNOILA, R.J. DI AUGUSTINE, R.P., MILLER, R.J. CHANG I. & CUATRECASAS F. 1978. An immunohistochemical and radioimmunological study of the distribution of met- and leu-enkephalin in the gastrointestinal tract. *Neuroscience* 3 1187-1196
- LORD J.A.H., WATERFIELD A.A., HUGHES J & KOSTERLITZ, H.W. 1977 Endogenous opioid peptides, multiple agonists and receptors. *Nature (Lond)* 267 495-499



- LUBINSKA, L. 1959 Region of transition between preserved and regenerating parts of myelinated nerve fibres. *J Comp Neurol* 113 315-335
- LUNDBERG J.M., AHLMAN H., DAHLSTRÖM A. & KEWENTER J. 1976 Catecholamine containing nerve fibres in the human abdominal vagus. *Gastroenterology* 70 516-522.
- LUNDBERG J.M., HÖKFELT T. SCHULTZBERG M. NORELL, G. NILSSON G. UVNÄS-WALLENSTEN K., TERENIUS L. DAHLSTRÖM A. REHFELD J.F. ELDE, R. & SAID S. 1978 a. Pathways of peripheral peptide neurons with special reference to the vagus nerve. *Neuroscience letters Suppl* 1 p. 224. Second European Neuroscience Meeting, Florence, Italy
- LUNDBERG J.M., HÖKFELT T. & NILSSON G. 1978 b. Peptide neurons in the vagus, splanchnic and sciatic nerves. *Acta Physiol Scand* 104 499-501
- LUNDBERG J.M., HÖKFELT T. ÅNGGÅRD A. UVNÄS-WALLENSTEN K. BRIMLIÖN S. BRODIN E. & FAHRENKRUG J. 1980 Peripheral peptide neurons. Distribution, axonal transport and some aspects on possible function. *Advances of biochemical psychopharmacology*. In *Neural peptides and neuronal communication* (ed E. Costa & M. Trabucchi) vol. 21. Raven Press, New York.
- LUNDBERG J.M. HÖKFELT T., SCHULTZBERG M. UVNÄS-WALLENSTEN K. KOHLER, C. & SAID S. 1979 Occurrence of vasoactive intestinal polypeptide (VIP)-like immunoreactivity in certain cholinergic neurons of the cat. Evidence from combined immunohistochemistry and acetylcholinesterase staining. *Neuroscience* 4 1539-1559
- MARTINSON J. & MUREN A. 1963 Excitatory and inhibitory effects of vagus stimulation on gastric motility in the cat. *Acta Physiol Scand* 57 309-316
- MARTINSON J. 1965 a. Vagal relaxation of the stomach. Experimental reinvestigation of the concept of the transmission mechanism. *Acta Physiol Scand* 64 453-462.
- MARTINSON J. 1965 b. The effect of graded vagal stimulation on gastric motility secretion and blood flow in the cat. *Acta Physiol Scand* 65 300-309
- MAY W.P. 1904-1901 The innervation of the sphincters and musculature of the stomach. *J Physiol* XXXI 262-271. XXVII 250
- McGILL, J.J. CAMERON A.J. HOBBSLEY M. & LeQUESNE L.P. 1969 The effect of fat upon gastric emptying after partial gastrectomy and after vagotomy with a drainage procedure. *Brit J Surg* 55 386.
- MIR, S.S. TELFORD G.L., MASON G.R. & ORMSBEE III H.S. 1979 Noncholinergic nonadrenergic inhibitory innervation of the canine pylorus. *Gastroenterology* 76 1443-1448
- MORGAN, K.G. SCHMALZ, P.F. & SZURSZEWSKI J.H. 1978 The inhibitory effects of vasoactive intestinal polypeptide on the mechanical and electrical activity of canine antral smooth muscle. *J Physiol (Lond)* 282 437-458
- MROZ, C.T. & KELLY K.A. 1977 The role of the extrinsic antral nerves in the regulation of gastric emptying. *Surg Gynec Obstet* 145 369-377

- MURYOBAYASHI T, MORI J., FUJIMARA, M. & SHIMAMOTO K. 1968 Fluorescence histochemical demonstration of adrenergic nerve fibres in the vagus nerve of cats and goats. *Jap J Pharmacol* 18: 285-293
- MÜLLER, G. 1963 Die funktionelle Anatomie des Magens. *Anat Anz* III (Erg) 298-311
- NICKERSSON M. & COLLIER, B. 1975 Drugs inhibiting adrenergic nerves and structures innervated by them. In *The pharmacological basis of therapeutics* (ed L.S. Goodman & A. Gilman) pp 533-564. MacMillan Publ Co Inc, New York.
- NILSSON G, PERNOW B., FISHER, G.H. & FOLKERS, K. 1975 Presence of substance P-like immunoreactivity in plasma from man and dog. *Acta Physiol Scand* 94: 542-544
- OPENCHOWSKI T. 1889 Über die nervösen Vorrichtungen des Magens. Experimentelle Studien. *Zentralbl Physiol Arch Anat* III: 1-10
- OTSUKA, M. & TAKAHASHI T. 1977 Putative peptide neurotransmitters. *Ann Rev Pharmacol Toxicol* 17: 415-439
- PAHLIN P.E. 1975 Extrinsic nervous control of the ileo-cecal sphincter in the cat. *Acta Physiol Scand Suppl* 426
- PAINTAL, A.S. 1954 A study of gastric stretch receptors. Their role in the peripheral mechanism of satiation of hunger and thirst. *J Physiol (Lond)* 126: 255-270
- PEASE D.C. 1969 Buffered formaldehyde as a killing agent and primary fixative for electron microscopy. *Anat Rec* 142: 342.
- POLAK, J.M., SULLIVAN S.N., BLOOM S.R., FACER, P. & PEARSE, A.G.E. 1977 Enkephalin-like immunoreactivity in the human gastrointestinal tract. *Lancet* May 977-974
- RATTAN S., SAID S.J. & GOYAL, R.K. 1977 Effect of vasoactive intestinal polypeptide (VIP) on the lower esophageal sphincter (LES) pressure. *Proc Soc Exp Biol Med* 155: 40-43
- RÜDINGER - 1879 Über die Muskelanordnung im Pfortner des Magens und am Anus. *Allg Wien Med Zeit* 4(1): 2-3 (?) 9-10
- SCHOFIELD G.C. 1968 Anatomy of muscular and neural tissue in the alimentary canal. *Handb Physiol* 4 (sect 6) 1579-1627
- SCHULTZBERG M., DREYFUS C.F., GERSHON M.D., HÖKFELT T., ELDE, R.P., NILSSON G., SAID S. & GOLDSTEIN M. 1978 VIP, enkephalin, substance P and somatostatin-like immunoreactivity in neurons intrinsic to the intestine: immunohistochemical evidence from organotypic tissue cultures. *Brain Res* 155: 239-248
- SCHULTZBERG M., LUNDBERG J.M., HÖKFELT T., TERENIUS L., BRANDT J., ELDE, R.P. & GOLDSTEIN M. 1978. Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. *Neuroscience* 3: 1169-1186
- SMITH C.L., KEWENTER J., CONNELL, A.M., ARDILL, J., MAYES R. & BUCKANAN K. 1975 Control factors in the release of gastrin by direct electrical stimulation of the vagus. *Amer J Dig Dis* 70: 13-22.

- SULLIVAN S., HUDA, A. & BASCHAS J.D. 1978 In vitro degradation of enkephalin. Evidence for cleavage at the gly-phe bond. *Communications in psychopharmacology* 2 525-531
- TELFORD G.L., MIR, S.S. MASON G.R. & ORMSBEE III H.S. 1979 Neural control of the canine pylorus. *Amer J Surg* 137 92-98.
- TERENIUS L. 1977 Opioid peptides and opiates differ in receptor selectivity. *Psycho-neuroendocrinology* 2 53-58
- TESCHEMACHIER, H. 1978 Endogenous ligands of opiate receptors (endorphins). In: *Developments in opiate research* (ed. A. Herz) chap 2. Marcel Dekker New York.
- THOMAS J.E. CRIDER, J.O. & MOGAN C.J. 1934 A study of reflexes involving the pyloric sphincter and antrum and their role in gastric evacuation. *Amer J Physiol* 108 683
- THOMAS J.E. & WHEELON H. 1922 The nervous control of the pyloric sphincter. *J Lab Clin Med* 7 375-391
- TORGERSEN J. 1942. The muscular build and movements of the stomach and duodenal bulb. *Acta Radiol Suppl* 45(1) 1-191
- UVNÄS-WALLENSTEN K., UVNÄS B. & NILSSON G. 1976 Quantitative aspects on the vagal control of gastrin release in cats. *Acta Physiol Scand* 96 19-28.
- UVNÄS-WALLENSTEN K. 1978 Release of substance P-like immunoreactivity into the antral lumen of cats. *Acta Physiol Scand* 104 464-468
- VALENZUELA, E.J. DEFILIPPI, C. & ATTILA C. 1976 Manometric studies on the human pyloric sphincter. *Gastroenterology* 70 481-483
- WERTHEIMER, E. 1891 Inhibition réflex du tonus et des mouvements de l'estomac. *Arch Physiol Norm Path (Paris)* 5(IV) 379-385
- VOLLE, R.L. & KOELLE G.B. 1975 Ganglionic stimulating and blocking agents. In: *The pharmacological basis of therapeutics* (ed. L.S. Goodman and A. Gilman) pp. 565-574. MacMillan Publ. New York.





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 487

I

# Diet and Muscle Metabolism in Man

with reference to fat and carbohydrate utilization and its regulation

By  
Eva Jansson

# L R R A T A

Page	present text	should be
6 line 28	5% protein	25% protoln
6 line 29	25% carbohydrates	5% carbohydrates
11 Fig 3	The values given for a-iv for FFA at rest should be multiplied by 1.7	
14 line 15	48 and 86%	63 and 101%
14 line 16	1.79	1.60
18 line 25	Fig 6	Fig 7
20 line 5	as measured at the of a 25 min	as measured at the <u>end</u> of a 25 min

ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 487

From the Department of Clinical Physiology  
Karolinska sjukhuset, Stockholm, Sweden.

# Diet and Muscle Metabolism in Man

with reference to fat and carbohydrate utilization and its regulation

By  
Eva Jansson

STOCKHOLM 1980





## CONTENTS

INTRODUCTION	5
MATERIAL AND METHODS	6
Subjects	6
Experimental procedures	6
Statistics	8
RESULTS AND COMMENTS	8
Muscle glycogen analyses	8
Respiratory exchange ratio	8
Intramuscular substrates and metabolites	9
Blood-borne substrates and metabolites	10
GENERAL DISCUSSION	13
Total leg metabolism	13
Aspects on citrate measurements	15
Metabolic regulation in relation to diet	16
Background	16
Rest	16
5 min exercise	17
25 min exercise	17
Females versus male subjects	18
CONCLUSIONS	20
Index of abbreviations	20
ACKNOWLEDGEMENTS	21
REFERENCES	22

The present thesis is based on the following papers which will be referred to by their Rosen numbers:

- I. JÄNSSON E. Acid soluble and insoluble glycogen in human skeletal muscle  
Acta Physiol Scand. Accepted for publication
- II JÄNSSON E. On the significance of the respiratory exchange ratio after different diets during exercise in man. Acta Physiol Scand. Accepted for publication
- III JÄNSSON E. & KALNER, L. Effect of diet on the utilisation of blood-borne and intramuscular substrates during exercise in man. Acta Physiol Scand. Accepted for publication
- IV JÄNSSON E. & KALNER, L. Effect of diet on muscle glycogen and blood glucose utilization during a short term exercise in man
- V JÄNSSON E. & KALNER, L. Leg citrate metabolism at rest and during exercise in relation to diet and substrate utilisation in man

In addition, some unpublished results are included.

## INTRODUCTION

Already in 1939 studies by Christensen and Hansen suggested that a change in the proportions of fat and carbohydrates in the diet would influence the relative oxidation of these two types of substrates in the working muscles as judged from the respiratory exchange ratio (R). By using the percutaneous muscle biopsy technique Bergström et al. (1967) and Gollnick et al. (1972) demonstrated a greater intramuscular glycogen content and a greater decrease in glycogen during exercise after a carbohydrate rich than after a fat rich diet. The present series of studies was undertaken in order to further investigate the relationship between the composition of the pre exercise diet and the substrate utilization during exercise thus suggested.

In the methods conventionally used for the analysis of intramuscular glycogen, perchloric acid extraction of the muscle tissue is a critical step. Thus variations in acid solubility of the glycogen present in the muscle is a potential source of error in these analyses. To evaluate the magnitude of this analytical problem a methodological study was performed (I).

R is a basic parameter used for evaluating a possible effect of the diet on the muscle substrate utilization during work in the present as well as in previous studies. However R gives only an indirect estimation of muscle substrate metabolism, since the gas exchange over the lungs represents a mean for the whole body. Furthermore extreme diets may cause general alterations of the acid base balance which would influence the release of  $\text{CO}_2$  from the bicarbonate pool and thereby change the R. Thus in a second study acid base parameters and the relationship between R and the respiratory exchange ratio as measured directly over the leg ( $\text{RQ}_L$ ) were studied during a submaximal exercise after two diets differing with respect to the relative proportions of fat and carbohydrates. This was done to evaluate if R gives a relevant estimation of the proportions of fat and carbohydrate oxidation in working skeletal muscle even after extreme diets.

To obtain a more detailed information on the contribution of blood-borne and intramuscular substrates during exercise after a fat and after a carbohydrate rich diet healthy subjects were studied during a long (III) and a short (IV) term submaximal exercise after the same types of diet as in the study just described. Thus in addition to intramuscular glycogen intramuscular triglycerides and blood-borne substrates such as free fatty acids (FFA) and glucose might also be influenced by the diet. An increased plasma concentration of FFA is probably at hand after a fat rich and carbohydrate poor diet and the utilization of free fatty acids (FFA) has been found to be related mainly to the plasma concentration of FFA (see e.g. Engenfeldt & Møhrén 1971). On the other hand a corresponding relationship between utilization and concentration has not been shown or thought to be at hand for glucose or glycogen utilization (cf Bendie & Baith 1958; Mørgen, Bendie & Engen 1959; Newsholme & Crabtree 1979).

Assuming that other factors than the supply of carbohydrates regulate the glycolysis concurrently with a varying availability of FFA the rate of fatty acid oxidation could possibly regulate the glycolysis by the action of metabolic intermediates on enzymatic steps (in the glycolysis) according to a hypothesis presented by Garland Randle & Newsholme (1963). Citrate was thus proposed to be a key-intermediate. This was based upon the findings that during an increased supply and oxidation of fatty acids in the perfused rat heart, an inhibition of the phosphofructokinase (PFK) reaction was found synchronously with an increased citrate content and that the PFK reaction in vitro was inhibited by citrate (Garland Randle & Newsholme 1963). Therefore the possibility of a citrate mediated regulation of the glycolysis due to an altered rate of fat oxidation was investigated by studying the leg citrate metabolism and its relation to the concomitant leg substrate utilization during exercise in the two dietary situations (V).

## MATERIAL AND METHODS

**Subjects** In 11 18 mal and 26 female healthy subjects participated. Mean and SD for various background variables are given in Table 1. All subjects were informed on the procedure and purpose of the experiment and on possible risks before giving an oral consent. The subject was free to withdraw their consent to participate at any time. The study was approved by the Ethical Committee of the Karolinska Hospital.

**Experimental procedure (I)** Musc glycogen analyses performed on tissue subjected to extraction by perchloric acid prior to the analyses were compared to corresponding analyses on tissue without such pretreatment. 99 biopsies covering wide range of glycogen concentrations were analysed. The muscle material was taken from the biopsies of study III and IV.

**Experimental procedure (II-V)** Each subject was studied at rest and during maximal bicycling exercise at 65% of maximal oxygen uptake ( $\dot{V}O_2$  max) on two occasions. The  $\dot{V}O_2$  max was determined before with the subjects on normal diet. The exercise time was 25 min in study II, III and V and 6 min in study IV. The first experiment was preceded by 5 days on fat rich and carbohydrate poor diet (~70% fat, 5% protein and 25% carbohydrate; 8 MJ/day). This was followed immediately by 3 days on a carbohydrate rich and fat poor diet (~70% carbohydrate, 10% fat and 20% protein; 8 MJ/day) and an identical experimental procedure. Details on the diet have been given in study II. On the days of the experiment the subjects came to the laboratory after light morning meal (~800 kJ) with the same composition as the preceding diet.

Table 1 Age, height, weight, maximal oxygen uptake ( $\dot{V}O_2$  max) and maximal heart rate (max HR) for the subjects in study II, III and IV (a number of subjects)

	Age	Height	Weight	$\dot{V}O_2$ max	Max HR	
	yr	cm	kg	l min <sup>-1</sup>	beats min <sup>-1</sup>	( )
	SD	SD	SD	SE	SD	
Study II	22	170	67	2.88	188	7 (2 M)
Study III	25	173	66	2.80	190	8 (9 M)
Study IV	28	171	67	2.8	190	8 (4 M)

The sampling for studies I and II was performed simultaneously on the subjects in studies III and IV. In studies III and V in addition to the present experimental procedure two groups of subjects were studied. In study III the analyses were performed in subjects after the fat and the carbohydrate diet and in study IV oxygen uptake and were analysed in 4 subjects after the two diets. These subjects (male and female) were of same age, height, weight and physical fitness as the other subjects.



Assuming that other factors than the supply of carbohydrates regulate the glycolysis concurrently with a varying availability of FFA the rate of fatty acid oxidation could possibly regulate the glycolysis by the action of metabolic intermediates on enzymatic steps (in the glycolysis) according to a hypothesis presented by Garland Randle & Newsholme (1963). Citrate was thus proposed to be a key-intermediate. This was based upon the findings that during an increased supply and oxidation of fatty acids in the perfused rat heart, an inhibition of the phosphofructokinase (PFK) reaction was found synchronously with an increased citrate content and that the PFK reaction in vitro was inhibited by citrate (Garland Randle & Newsholme 1963). Therefore the possibility of a citrate mediated regulation of the glycolysis due to an altered rate of fat oxidation was investigated by studying the lag citrate metabolism and its relation to the concomitant lag substrate utilization during exercise in the two dietary situations (V).

## MATERIAL AND METHODS

**Subjects** In all 18 male and 26 female healthy subjects participated. Mean and SD for various background variables are given in Table I. All subjects were informed on the procedure and purpose of the experiment and on possible risks before giving an oral consent. The subjects were free to withdraw their consent to participate at any time. The study was approved by the Ethical committee of the Karolinska Hospital.

**Experimental procedure (I)** Muscle glycogen analyses performed on tissue subjected to extraction by perchloric acid prior to the analyses were compared to corresponding analyses on tissue without such pretreatment. 99 biopsies covering a wide range of glycogen concentrations were analysed. The muscle material was taken from the biopsies of studies I, III and IV.

**Experimental procedure (II-V)** Each subject was studied at rest and during ergometric bicycle exercise at 65% of maximal oxygen intake ( $\dot{V}O_2$  max) on two occasions. The  $\dot{V}O_2$  max was determined beforehand with the subjects on normal diet. The exercise time was 25 min in studies II, III and V and 6 min in study IV. The first experiment was preceded by 3 days on a fat rich and carbohydrate poor diet (~70% fat, 3% protein and 25% carbohydrate; 8 MJ/day). This was followed immediately by 5 days on carbohydrate rich and fat poor diet (~70% carbohydrate, 10% fat and 20% protein; 8 MJ/day) and an identical experimental procedure. Details on the diet have been given in study II. On the days of the experiments the subjects came to the laboratory first in the morning meal (~800 kJ) with the same composition as the preceding diet.

Table 1 Age, height, weight, maximal oxygen uptake ( $\dot{V}O_2$  max) and maximal heart rate (max HR) for the subjects in study II, III and IV (n = number of subjects)

	Age		Height		Weight		$\dot{V}O_2$ max		Max HR		( % )
	yr	SD	m	SD	kg	SD	l min <sup>-1</sup>	SD	beats min <sup>-1</sup>	SD	
Study II	32	3	170	11	67	11	2.03	0.42	190	9	7 (2-5)
Study III	38	3	172	8	64	7	3.00	0.65	190	18	20 (9-31)
Study IV	24	3	177	8	67	5	3.10	0.88	190	9	7 (4-31)

1) The sampling for studies I and V was performed simultaneously on the subjects in studies III and IV. In study III and in addition to the present experimental procedure two groups of subjects were studied. No analyses were performed in 6 subjects after the fat and the carbohydrate diet and in study V oxygen uptake and R were analysed in 4 subjects after the two diets. These subjects (males and females) were of same age, height, weight and physical fitness as the other subjects.

PCA insoluble glycogen  
nmol/kg dwt

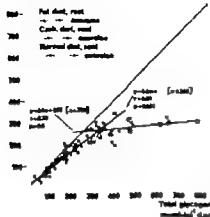


Fig. 1 Relationship between glycogen concentration in PCA pretreated muscle samples (PCA insoluble glycogen) and glycogen in sample without acid pretreatment (total glycogen). The larger symbol represent samples where PCA insoluble and total glycogen were determined simultaneously.

*Intramuscular substrates and metabolites (III, IV, V; Fig. 2)* The muscle glycogen concentration was lower both at rest and during exercise after the fat than after the carbohydrate diet and the decrease in glycogen concentration during exercise tended to be lower after the fat diet. The interindividual difference in glycogen decrease was larger after the carbohydrate than after the fat diet. The intraindividual variation in glycogen concentration before and after exercise was larger after the carbohydrate diet (III). However, it could not be concluded whether there really was a large variation between subjects or legs in decreases in the whole muscle, or if the differences were caused e.g. by variations in glycogen between different parts of the muscle. The large variation could anyway have contributed to the lack of a significant difference between the diets in glycogen decrease. Support for the idea that the decrease was actually smaller after the fat than after the carbohydrate diet was found in the fact that the muscle lactate accumulation as well as the lactate release over the legs were smaller both in the early phase and at the end of exercise.

There was no significant decrease in intramuscular triglyceride concentration during exercise after any of the diets. This may be due to the large variations in fat distribution in the muscle tissue (Carlsson, Ekblom & Fröberg 1971; Ekblom 1978). Other studies have shown a breakdown of muscle triglycerides during exercise of both short and long duration (Ekblom 1977) and numerically the difference between triglyceride concentrations at the end and at the start of exercise was negative after the fat and positive after the carbohydrate diet. This could be expected if the utilization of intramuscular triglycerides is greater after a fat than a carbohydrate diet.

The muscle citrate concentration was higher after the fat than after the carbohydrate diet at rest and 6 min of exercise, whereas no difference was found at the end of exercise. The glucose-6-phosphate (G-6-P) concentration was higher at rest, tended to be higher at 6 min of exercise and was lower at the end of exercise after the fat than after the carbohydrate diet.



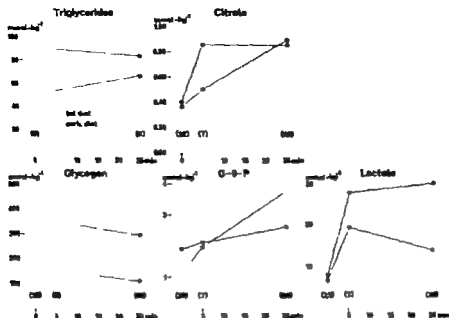


Fig 2 Muscle concentrations of triglyceride, glycogen, citrate, glucose-6-phosphate (G-6-P) and lactate at rest and after 6 and 25 min of exercise after the fat (filled circles) and after the carbohydrate (open circles) diet. The number of subjects for each point of time and analysis is given within parentheses.

- 1) The glycogen values at 6 and 25 min were calculated from the mean pre-exercise value (23) subtracted by median decrease over the 6 min work period (6) and mean post-exercise value (23) subtracted by median decrease over the 25 min work period (16) respectively.

**Blood-borne substrates and metabolites (III-IV)** During exercise the estimated leg blood flow was of similar magnitude in both dietary situations and consequently the difference between diets in uptake or release was mainly the same as estimated from the a-fv differences alone. However, at rest, due to the higher estimated leg blood flow after the fat diet, the uptake/release was not directly reflected by the a-fv differences (see V).

The plasma FFA concentration as well as the FFA uptake were higher after the fat than after the carbohydrate diet both at rest (unpublished) and during exercise (Fig. 3).

It has repeatedly been demonstrated that the uptake of FFA from blood to muscle is correlated with the arterial FFA concentration and that the fractional extraction is unaltered or slightly decreased when the FFA concentration increases (e.g. Hagenfeldt & Wahren 1971). In contrast to this, in the present study the fractional extraction during exercise tended to be higher at an increased FFA concentration. Thus, the gross extraction of FFA was on the average 82% greater after the fat than after the carbohydrate diet, whereas the arterial FFA concentration was only 66% higher. The present finding could possibly indicate a stimulation of intracellular fat utilization in exercising muscle after a fat-rich diet in excess of the effect of an increased availability.

The arterial glucose concentration was lower after the fat than after the carbohydrate diet both at rest and during exercise (Fig 3). The uptake of glucose at rest was also lower but during exercise no significant diet related difference was found. In fact there was a tendency to greater uptake after the fat diet (Fig 3).

The glucose extraction during exercise was not lowered after the fat diet in spite of an increased FFA extraction and presumably also an increased FFA oxidation. In contrast it should be noted that an inverse correlation between FFA and glucose extraction has been observed for the myocardium (Lassus et al 1971). However the present finding is in agreement with those of previous studies on the effect on the skeletal muscle metabolism of FFA-lowering by nicotinic acid which did not alter the glucose extraction by the working forearm (e.g. Rajzer et al 1978). On the other hand

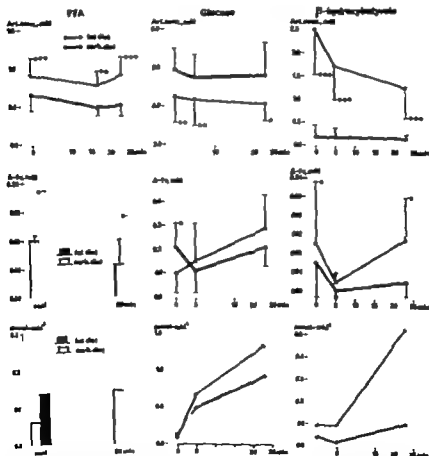


Fig 3 Arterial concentrations, arterial-venous (a-f) differences (gross extraction for FFA) and uptake of plasma free fatty acids (FFA), blood glucose and B-hydroxybutyrate at rest and during exercise for the fat (filled line / circles/bar) and after the carbohydrate (open line / circles / bars) diet.

there was a tendency towards an inverse relationship between the glucose extraction and the glycogen concentration in the muscle after the fat diet. This is in agreement with the observation of a higher glucose extraction over one leg with low muscle glycogen than over one with high muscle glycogen content (Gollnick et al 1980)

The arterial  $\beta$ -hydroxybutyrate concentration was higher after the fat diet both at rest and during exercise (Fig 3) At rest there was a significant uptake only after the carbohydrate diet and at the end of the exercise only after the fat diet

$\beta$ -hydroxybutyrate may be utilized in the oxidative metabolism. However the exchange of acetoacetate over the exercising leg was not measured. Consequently it is not known whether or not the  $\beta$ -hydroxybutyrate was fully oxidized. However if so the contribution to the total oxidative metabolism could have been about 8% after the fat diet as compared to 2% after the carbohydrate diet and could thus have contributed only moderately to the difference between the diets in substrate utilization.

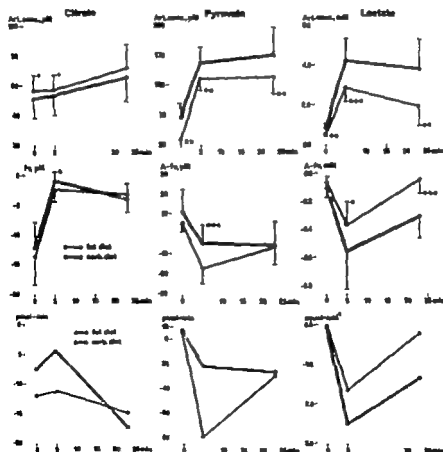


Fig 4 Arterial concentrations, arterial-venous (a-v) differences and uptake of blood lactate, pyruvate and lactate after the fat (filled line and circles) and after the carbohydrate (open line and circles) diet

The arterial lactate and pyruvate concentrations were lower after the fat than after the carbohydrate diet both at rest and during exercise (Fig. 4). At rest the lactate release did not differ between the diets but during exercise it was smaller after the fat diet (Fig. 4). Pyruvate was taken up at rest whereas after 5 min of exercise there was a release after both diets but the release was larger after the fat diet in contrast to the situation for the lactate release which was smaller after the fat diet. At the end of exercise no significant difference between the diets in pyruvate release was found.

The arterial citrate concentration was higher after the fat than after the carbohydrate diet both at rest and during exercise. There was a net release of citrate after both diets both at rest and during exercise. At rest and 5 min of exercise the citrate release was greater after the fat diet whereas at end of exercise no diet related differences were found.

Notably the greater release of pyruvate and citrate found after the fat than after the carbohydrate diet, occurred together with a lower lactate release. This indicates an inhibition of the pyruvate dehydrogenase (PDH) reaction, directly or indirectly coupled to the increased citrate formation.

#### GENERAL DISCUSSION

**Whole leg metabolism.** The simultaneously measured extractions of oxygen and blood-borne substrates makes it possible to estimate the relative net contribution of these substrates to the total oxidative metabolism and if R is taken into account, also of the fat and carbohydrate oxidation, respectively (Fig. 5).

After 25 min of exercise FFA contribution to the total oxidative metabolism was 24% after the fat diet and 14% after the carbohydrate diet. The remaining fat oxidation, 39% of the total after the fat and 13% after the carbohydrate diet, must consequently have been covered by plasma and intramuscular triglycerides. Assuming an active muscle mass in the legs of 6 kg (cf. Esseen, Bogenfaldt & Kaijser 1977) the pre-exercise concentrations after both diets suggested a 10-20 times greater supply of intramuscular triglycerides ( $90.7 \text{ mmol} \times \text{kg}^{-1}$  after the fat diet and  $50.4 \text{ mmol} \times \text{kg}^{-1}$  after the carbohydrate diet) than that required to cover all the remaining fat oxidation but in no dietary situation was a significant decrease noted. The tendency towards a decrease in concentration after the fat but not after the carbohydrate diet may suggest, however, that more intramuscular triglycerides are utilized after the fat diet. Nothing is to our knowledge known about the plasma triglyceride extraction during exercise after different types of diet, although after a normal mixed diet it could cover at the most 25% and probably far less of the oxidative metabolism (Olsson et al. 1975).

The glucose extraction at 25 min of exercise was equivalent to 27 and 21% of the oxidative metabolism after the fat and the carbohydrate diet respectively but when subtracting the lactate release the glucose contribution would change to 25% after

there was a tendency towards an inverse relationship between the glucose extraction and the glycogen concentration in the muscle after the fat diet. This is in agreement with the observation of a higher glucose extraction over one leg with low muscle glycogen than over one with high muscle glycogen content (Gollnick et al. 1980).

The arterial  $\beta$ -hydroxybutyrate concentration was higher after the fat diet both at rest and during exercise (Fig. 3). At rest there was a significant uptake only after the carbohydrate diet and at the end of the exercise only after the fat diet.

$\beta$ -hydroxybutyrate may be utilized in the oxidative metabolism. However the exchange of acetoacetate over the exercising leg was not measured. Consequently it is not known whether or not the  $\beta$ -hydroxybutyrate was fully oxidized. However if so the contribution to the total oxidative metabolism could have been about 8% after the fat diet as compared to 2% after the carbohydrate diet and could thus have contributed only moderately to the difference between the diets in substrate utilization.

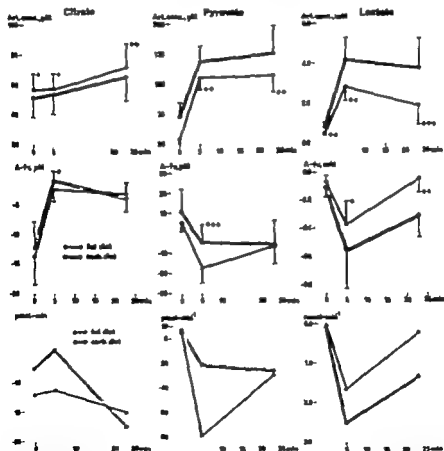


Fig. 4 Arterial and arterio-venous differences and uptake of blood lactate, pyruvate and lactate after the fat (filled line and circles) and after the carbohydrate (open lines and circles) diet.

Thus the general picture suggests that (1) a fat rich diet increases the relative contribution of fat to the oxidative metabolism during exercise as compared to a carbohydrate rich diet, (2) the increased fat utilization is to a great extent covered by plasma FFA and (3) the simultaneous decrease in carbohydrate utilization concerns muscle glycogen rather than glucose.

At rest the situation seemed quite different as compared to that during exercise. There was an uptake of substrates in excess of the oxidative utilization and this indicated a net increase of the stores of especially fat and to some extent carbohydrates.

*Aspects on citrate measurements* A number of technical problems are associated with the estimation of muscle citrate concentrations and some of them will be discussed below. Firstly a rapid increase of muscle citrate occur at cessation of work (unpublished). Most biopsies were taken and frozen within 10 s after the last muscle contraction. The citrate increase in the muscle over this period of time is difficult to estimate. However an increase of about 30% from the 10th to the 20th s after a submaximal exercise with the subjects on a normal diet has been observed (unpublished).

Secondly human skeletal muscle is composed of a mixture of type I (red) and type II (white) muscle fibres in a ratio of about 1:1 but a relatively large variation may occur within an individual and within a particular muscle. This together with the fact that the citrate content is approximately twice as high in the slow twitch fibres as in the fast twitch fibres in human muscle both at rest and immediately after exercise contributes to the methodological error of citrate estimations (cf Easte 1978).

Thirdly animal studies have shown an accumulation of citrate in red but not in white skeletal muscle with an increased rate of fat oxidation during exercise (Bennie Winder & Holloszy 1976). Thus to evaluate in greater detail the role of citrate in the regulation of muscle substrate utilization analyses should be carried out on individual red and white muscle fibres. However this was not done in the present study.

Finally the critical concentration of citrate for the regulation of glycolysis is the concentration in the sarcoplasm and not the total muscle citrate. Excess citrate produced in the mitochondria will be transported to the sarcoplasm and from there to the extracellular space. The release of citrate from the muscle may therefore better than the total muscle concentration reflect the sarcoplasmic citrate.

The present study demonstrated a net release of citrate from the leg to the blood both at rest and during exercise after both diets. The turnover of citrate in relation to its concentration in blood and muscle was high which allows rapid changes in concentrations which in turn is a prerequisite for citrate's regulatory importance. A good correspondence between the estimated citrate release and the muscle citrate concentration was found after both diets as well as diet related differences. Thus in spite of the great technical difficulties described above in estimating the actual sarcoplasmic citrate concentration the found differences between the diets support citrate being of regulatory importance for fat and carbohydrate metabolism.

*Metabolic regulation in relation to diet Background.* (Fig 6) According to recent theories (Newsholme & Crabtree 1979) glycolysis in skeletal muscle may be divided into two separate pathways (1) glycolysis-from-glycogen and (2) glycolysis-from-glucose, on the basis of different flux generating steps (i.e. the reaction where the flux through the pathway is initiated). The capability of the muscle to control these two flux generating reactions might differ. Thus phosphorylase (PL) catalyses the flux generating step for the flux from muscle glycogen (the pathway substrate). The PFK reaction has a potential to regulate the activity of PL via the G-6-P concentration. However only PLb and not PLa is thought to be under the control of PFK (Morgan & Permezziani 1964) and PFK may therefore only have a partial capacity to regulate the flux generating reaction. The substrate for the second pathway glycolysis-from-glucose is under debate. It has been argued (Newsholme 1977) that the pathway substrate is in fact liver glycogen and if so glucose would be regarded as a metabolic intermediate. It has further been considered unlikely that muscle PFK, by varying the G-6-P level which in turn influences the hexokinase (HK) reaction in the muscle could regulate the suggested flux generating reaction in the liver. The glucose transport over the muscle cell membrane has instead been proposed to be a reaction that could influence the hepatic phosphorylase via (blood) glucose itself (Newsholme & Crabtree 1979). Thus according to the theories now presented citrate by its influence on the PFK reaction might have a potential to regulate only the rate of glycolysis-from-glycogen and only via PLb inhibition. In an attempt to apply this approach to the findings in the present study the resting 5 min and 25 min situations will be discussed separately.

*Rest:* The leg citrate release as well as muscle citrate and G-6-P were found to be higher after the fat than after the carbohydrate diet. An estimation of the quantitative relationship between total rate of glycolysis after the diets as judged from R and exchange of lactate and pyruvate over the legs suggests a lower rate of glycolysis after the fat diet. The measured substrate utilization revealed a lower glucose and

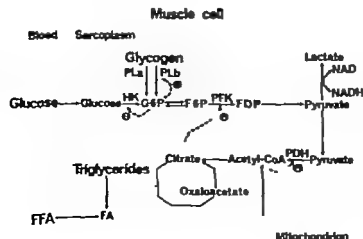


Fig 6 A schematic illustration of metabolic pathways involved in the degradation of fat and carbohydrate in the muscle. II Cont 1 of the glycolysis and pyruvate oxidation by citrate and acetyl-CoA.

a higher PFK uptake after the fat than after the carbohydrate diet. This supports the idea of a lower rate of glycolysis as proposed above and consequently it appears possible that the inhibition of glycolysis could be mediated through the increased muscle citrate acting via a PFK inhibition: an increased G-6-P level and an inhibition of Pfk.

*5 min exercise* The situation at 5 min of exercise showed some features in common with that at rest. Both citrate release over the leg and the muscle citrate concentration were higher after the fat than after the carbohydrate diet and the G-6-P concentration showed the same tendency. The total rate of glycolysis as judged from  $\Sigma$  lactate, pyruvate and alanine release was lower after the fat diet also during exercise. Support for this was found in the lower ( $p < 0.3$ ) muscle glycogen decrease and lower muscle lactate increase after the fat diet. However the glucose uptake did not differ between the diets. Accordingly the increased muscle citrate and G-6-P concentrations are consistent with the theory of a citrate mediated inhibition of glycolysis including a control of the first generating step through the G-6-P level. The flux of glucose through the glycolysis seemed not to be different in the two dietary situations as could be expected if PFK is not a regulatory enzyme in the glycolysis-from-glucose (cf. Wassholme & Crabtree 1979). Thus it is suggested that the glycolysis is partly under control of the PFK reaction. The observation of a greater release of pyruvate by the leg after the fat than after the carbohydrate diet in spite of a lower lactate release indicates however an imbalance between the rate of glycolysis on one hand and the PDH availability and PDH activity on the other. One factor contributing to this suggested imbalance might be an increased rate of glycolysis caused by an increased conversion of Pfb into Pfa. This view is supported by our earlier finding (Jansson, Bjørklund & Kalljær 1980) of increased plasma catecholamine levels after a similar fat diet with ensuing  $\beta$ -receptor stimulation and thereby increased formation of cyclic AMP which might in turn stimulate a conversion of Pfb into Pfa (Dunforth & Balazs 1964; Butherland, Oye & Butcher 1965). Support for a difference between the diets in PDH availability was found in the lactate/pyruvate ratio in the blood which was lower after the fat diet. Furthermore animal studies have demonstrated a PDH inactivation after a high rate of fat oxidation (Barnick, Löffler & Wieland 1975).

*15 min exercise* At this time diet related differences were found neither for the citrate release nor for the muscle citrate concentration. However the total rate of glycolysis estimated as described above was still lower after the fat diet. The measured substrate utilizations partly support this (higher PFK uptake, tendency to lower decrease in glycogen, lower increase in lactate and a tendency to higher glucose uptake). The G-6-P concentration was found to be lower after the fat diet. Thus no correspondence was found between muscle citrate accumulation and the rate of glycolysis at the end of exercise. However after a work period of this duration it cannot be excluded that the lower rate of glycolysis after the fat diet would merely be related to very low glycogen concentrations. Thus in recent studies on short term intense exercise and lactate accumulation it has been suggested that the glycogen concentration



would influence the rate of glycogenolysis because the PL may not be saturated with its substrate (Kilbussen & Bjørgaard 1980 I Jacobs and J Karlsson personal communication)

The finding of similar magnitudes of both citrate release and muscle citrate concentration when comparing the dietary situations indicated that the rates of citrate synthesis were the same. The latter is in turn dependent on the supply of acetyl-CoA and oxaloacetate. The similar citrate magnitude might thus be explained as follows:

The amount of acetyl-CoA, as derived from fatty acids, was probably larger after the fat than after the carbohydrate diet. However the amount of acetyl-CoA as derived from carbohydrates was possibly markedly reduced after the fat diet because of the very low levels of glycogen that were at hand. The supply of oxaloacetate might as well have been reduced. Due to this it is possible that neither the total supply of acetyl-CoA nor the rate of citrate synthesis differed between the diets or that a possibly reduced supply of oxaloacetate was a limiting factor for the citrate synthesis after the fat diet.

*Female versus male subjects* (Fig 7 Table 3) The group of subjects was not a real random sample which actually is a prerequisite for making a relevant comparison between the sexes. However in spite of this a rough comparison was made. Thus among the background variables height, weight,  $\dot{V}O_2$  max and the relative proportions of different muscle fibre types in m. quadriceps femoris (unpublished) differed significantly between the sexes. There was a higher proportion of type I fibres in the quadriceps muscle of the females whereas in earlier studies on sex differences in relative fibre proportions a lower percentage of type I fibres in females (Koss & Karlsson 1979) or no differences at all (Radberg & Jansson 1976) have been observed. The general picture given by certain metabolic variables (see Fig 6) indicated a tendency towards a relatively higher fat oxidation in the female than in the male subjects as indicated e.g. by the tendencies towards lower R and higher oxygen uptake in relation to  $\dot{V}O_2$  max. Also a possible sign of higher activities in the

Table 3 Age, height, weight, maximal oxygen uptake ( $\dot{V}O_2$  max) and maximal heart rate (Max HR) of the 9 male and female subjects and the relative proportions of the 4 recent muscle fibre types in % of the total in the female subjects

Variable	Males		Female		Females/male ratio	Significance of the male/female difference
	no	%	no	%		
Age, yr	24	3	2	3	0.0	
Height, cm	86		65	7	1.09	0.001
Weight, kg	73	6	6		1	0.00
$\dot{V}O_2$ max l min	3.46		2	39	32	0.0
Max HR beats min	18		9		0.90	
Type I fibres %	6		56		0.1	0.0
Type IIa fibres	35		34	0.4	35	0.1
Type IIb fibres			5.5		0.0	
Type IIc fibres	9		2.3			n.s.

1) the subgroup of subjects on which fibre types were available. The sex differences regarding the background variables were of the same magnitude as in the total sample

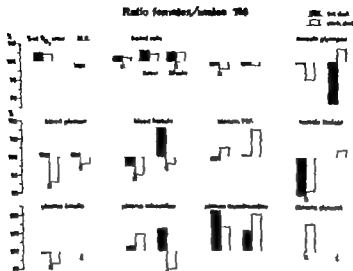


Fig 7 The ratio female/male subject  $\times 100$  of the oxygen uptake ( $\text{VO}_2$ ) and mechanical efficiency (M.E.) during exercise heart rate respiratory exchange ratio (R) plasma FFA, blood glucose blood lactate muscle glycogen plasma lactate plasma insulin plasma adrenaline and plasma noradrenaline at rest and during exercise after the 1st (filled bars) and after the 2nd (open bars) diet. The elevations of the different ratios were based on mean values for all subjects on which the different analyses were performed. 1) The insulin, adrenaline, noradrenaline and glycerol values were performed on Jensen, Bjørnsdal & Kaijser (1980) and measurements were performed on 2 male and 5 female subjects only.

sympathetic nervous system was found in the tendencies towards higher level of plasma catecholamines, higher heart rate and lower plasma insulin concentration in the female subjects. These findings seem reasonable if considering the fact that the females have a relatively larger amount of body fat than the males and that the catecholamines and insulin are both important hormones in the regulation of lipolysis (for ref. see Neuhof & Stærk 1973). To our knowledge only a few studies have been performed with the aim of comparing the sexes with respect to differences in the relative oxidation of fat and carbohydrates. One study comparing well trained female and male runners gave no support for a sex related difference in fat metabolism (Ostili et al. 1979). Alternatively the tendencies towards a relatively higher fat metabolism in the females might exclusively be related to the fact that the females showed a higher percentage of type I fibres which have higher capacity to oxidize fat than type II fibres (S. & Rasmussen et al. 1973).

## CONCLUSIONS

1. Muscle glycogen consists of fractions with different acid solubility. The more acid soluble fractions constitute a greater proportion of total glycogen at high than at low concentrations. This is a source of error when glycogen analyses are performed on perchloric acid extracted tissue.
2. The respiratory exchange ratio (R) as measured at the end of a 25 min submaximal exercise does very likely estimate the proportions of fat and carbohydrate oxidation in skeletal muscle both after a fat rich and after a carbohydrate rich diet.
3. A fat rich diet increases the relative contribution of fat to the oxidative metabolism during submaximal exercise. This increase is, to a great extent, covered by fatty acids from plasma. The concomitant decrease in carbohydrate utilization concerns muscle glycogen rather than glucose.
4. The inhibition of glycolysis seen at rest and during the early phase of submaximal exercise after a fat diet, might be due to increased intramuscular citrate acting on the phosphofructokinase reaction. In addition, the pyruvate dehydrogenase reaction is possibly inhibited during the fat diet conditions. The inhibition of glycolysis, seen at the end of exercise after the fat diet, does not seem to be citrate mediated.

## Index of abbreviations

FFA	free fatty acid	PDH	pyruvate dehydrogenase
FA	fatty acids	PFK	phosphofructokinase
F-6-P	fructose-6-phosphate	PL	phosphorylase
F-1,6-D-P	fructose-1,6-diphosphate	PLa	phosphorylase a
G-6-P	glucose-6-phosphate	PLb	phosphorylase b
HK	hexokinase	R	respiratory exchange ratio over the lungs
NAD	nicotinamide adenine dinucleotide (oxidized form)	R <sub>Q</sub> <sub>L</sub>	piratory exchange ratio over the lungs as measured in the blood
NADH	nicotinamide adenine dinucleotide (reduced form)		

## ACKNOWLEDGMENTS

This work was performed at the Departments of Clinical Physiology at Karolinska Institute and Södersjukhuset. It is a pleasure for me to thank the following who have helped me in different ways:

Professor Bengt Pernow for placing the laboratory facilities at my disposal for support and valuable advice. Professor Lars Böttger who introduced me to biochemistry. Professor Bengt Saltin for introducing me to the field of exercise physiology and for constructive criticism and valuable support. Professor Professor Lennart Kaijser for his inspiring teaching guidance and personal support during the progress of the work. Professor Lars Carlsson for placing the resources of Qufast V Research Institute at my disposal. Professor Lennart Kaijser for valuable criticism. Assistant Professors Lars Hagendahl, Alf Ström-Karlsson and Lars-Olof Rönnevi for valuable advice and criticism during the work of the manuscripts.

Doc Birgitta Engström, Jan Henriksson, Ina Jacobs, Bert Sköld and others for stimulating discussions during the study and the preparation of the manuscript.

Barbara Almqvist, Ingela Broberg, Britt Elving, Maria Hallberg, Gunilla Margareta Nilsson, Dragica Ojurovic, Annette von Scheele, Helena Sjöström for excellent technical assistance and stimulating collaboration.

Inga Jansson for skilful drawing of the figures. The entire staff of the Department of Clinical Physiology without whose kind help and interest these studies could not have been accomplished. Margaretha Palmberg and Gerlith Petersson for excellent secretarial help and most valuable advice and support in the preparation of the manuscripts. All volunteers who have participated in the investigation.

The study was supported by grants from the Karolinska Institute, the Swedish Research Council (No. 4354) the Research Council of the Swedish Sports and Recreation Commission, and the Coca-Cola Export Corporation, Sweden.

SCHOLANDER P F 1947 Analyze for accurate estimation of respiratory gases in one half cubic centimeters samples J Biol Chem 167:233-250

SEVERINGHAUS J W & BRADLEY A F 1958 Electrode for blood  $pO_2$  and  $pCO_2$  determination J Appl Physiol 13:515-520

SUTHERLAND E W ØYE I & BUTCHER R W 1965 The action of epinephrine and the role of the adenyl cyclase system in hormone action Rec Progr Horm Res 21:623-646

## ACKNOWLEDGEMENTS

This work was performed at the Departments of Clinical Physiology at Karolinska sjukhuset and Serafimerlasarettet. It is a pleasure for me to thank all those who have helped me in different ways

Professor Bengt Pernow for placing the laboratory facilities at my disposal and for support and valuable advice. Professor Lars Ernster who introduced me into biochemistry. Professor Bengt Saltin for introducing me to the field of research in physiology and for constructive criticisms and valuable support. My co-author Professor Lennart Kaijser for his inspiring teaching guidance and personal support. During the progress of the work, Professor Lars Carlsson for placing the laboratory resources of Gustaf V Research Institute at my disposal. Professor Richard Edwards for valuable criticisms. Assistant Professors Lars Hagenfeldt, Alf Holmgren, Jan Karlsson and Lars-Olof Ronnevi for valuable advice and criticisms during the preparation of the manuscripts.

Dns Birgitta Enein, Jan Henriksson, Iris Jacobs, Kent Sahlin and Rickard Schalis for stimulating discussions during the study and the preparation of the manuscripts. Barbara Almqvist, Ingela Broberg, Britt Elving, Maria Rollind, Gunilla Medalen, Margareta Nilsson, Dragica Ojurovic, Annette von Schewelov, Helena Skoglund-Mallberg for excellent technical assistance and stimulating collaboration.

Inga Jönner for skilful drawing of the figures. The entire staff at the Departments of Clinical Physiology without whose kind help and interest these studies would never have been accomplished. Margaretha Palmfö and Gurlith Petersson for their excellent secretarial help and most valuable advice and support in the preparation of the manuscripts. All volunteers who have participated in the investigation.

The study was supported by grants from the Karolinska Institute, the Swedish Medical Research Council (No. 4554), the Research Council of the Swedish Sports Federation and Coca-Cola Export Corporation, Sweden.

## REFERENCES

- BASSO L V & HAVEL R J 1970 Hepatic metabolism of free fatty acid in normal and diabetic dog J Clin Invest 49 337-347
- BROGSTROM J 1962 Muscle electrolyte in man Scand J Clin Lab Invest Suppl 68
- BROGSTROM J, HERMANNEN L, HOLTHAN E & SALTIN B 1967 Diet muscle glycogen and physical performance Acta Physiol Scand 71:140-150
- BUCHER, T, CROK, R, LAMPRECHT W & LATZER E 1962 Pyruvat In Methoden der Enzymatischen Analyse, 2. Aufl. Verlag Chemie Weinheim
- CARLSSON L.A., EKELUND L.-G. & FRÖBERG S O 1971 Concentrations of triglycerides, phospholipid and glycogen in skeletal muscle and of free fatty acids and  $\beta$ -hydroxybutyric acid in man in response to exercise, Europ J Clin Invest 1 248-254
- CHERNICK, S S 1969 Determination of glycogen and acylglycerol Methods in Enzymology 14:627-630
- CHRISTENSEN E H & HANSEN O 1939 III Arbejdsfædhed og Ernæring Skandinav Archiv 81:1-12
- COSTILL, D L, FINK W J, GUTCHELL L W, IVY J L & WITTMANN P A 1979 Lipid metabolism in skeletal muscle of endurance-trained male and female J Appl Physiol 47:787-791
- DANFORTH W B & HELMKEITH E 1964 Regulation of glycogenolysis in muscle I The conversion of phosphorylase B to phosphorylase A in frog aorta muscle J Biol Chem 239:3133-3138
- DRABKIN E L & SHIMDT C F 1943 Spectrophotometric studies J Biol Chem 157:69-83
- ESSEN B 1977 Intramuscular substrate utilization New York Academy of Sciences 301:30-44
- ESSEN B 1978 Studies on the regulation of metabolism in human skeletal muscle using intermittent release as an experimental model Acta Physiol Scand Suppl 454
- ESSEN B, HAGENFELDT L & KALLJÖR L 1977 Utilization of blood-borne and intramuscular substrates during continuous and intermittent exercise in man J Physiol (London) 265 489-507
- FUSÉN B, JANSSON E, HENRIKSSON J, TAYLOR A W & SALTIN B 1975 Metabolic characteristics of fibre types in human skeletal muscle Acta Physiol Scand 95: 153-165
- GARLAND P B, RANDLE P J & NEUHOLME R A 1963 Citrate as an intermediary in the inhibition of phosphofructokinase in the heart muscle by fatty acids, ketone bodies, pyruvate, diabetogenic and starvation Nature 200 169-170
- GOLLIWICK P D, FIEHL K, SAUBERT IV C W, ARMSTRONG R B & SALTIN B 1972 Diet, exercise and glycogen changes in human muscle fibers J Appl Physiol 33:421-425
- GOLLIWICK P D, PERROW B, ESSEN B, JANSSON E & SALTIN B 1980 Availability of glycogen and plasma FFA and substrate utilization in leg muscle of man during exercise Clinical Physiology Submitted for publication
- HAGENFELDT L & WAHREN J 1971 Metabolism of free fatty acid and ketone bodies in skeletal muscle I Muscle metabolism during exercise (ed B Pernow and B Saltin) pp 153-163 Plenum Press New York
- HEDBERG G & JANSSON E 1976 Skeletal muscle fiber distribution capacity and its effect in different physical activities among students in high school Pedagogiska rapport nr Omf. No 54 (in Swedish; English summary)
- HOCKEY G, LÜFFLER G & WIELAND O H 1975 Active and inactive forms of pyruvate dehydrogenase in skeletal muscle related to the metabolic and functional state of the muscle II Fed Proc 54 142-145
- HO, R J 1970 Radiochemical assay of long chain fatty acid using  $^{63}\text{Ni}$  tracer Analyt Biochem 34 55-113

- MORFET M J 1962 L( ) Lactatbestimmung mit Lactat Dehydrogenase und DFM In Methoden der Enzymatischen Analyse pp 266-270 Verlag Chemie Weinheim
- MUGGETT A S T G & WILSON D A 1957 U r glucose oxidase pe oxidase and o-dianilidide in d t rination f blood and urinary glucose Lancet 2 368 370
- JANSSON E, RICHARDSON, P & KALLNER L 1980 Di t induced changes in sympathetic activity in relation to substrate utilization during submaximal exercise in man In manuscript
- JORFELDT L & WARREN J 1971 Leg blood flow during exercise in man Clin Sci 41: 459-473
- KALLNER, L, NYE R R, EKLUND B, OLSSON A G & CARLSON L A 1978 The r l tion between carbohydrate ext action by the forearm and arterial f ee f tty a id concent tion in man I Forearm work with isotonic acid infusion Scand J Clin Lab Invest 38:41-47
- KARLSSON J 1971 Lactate and phosphagen concentrations in working muscle of man Acta Physiol Scand Suppl 358
- KLAUDEN K. & RINGDAHL G 1980 Glycogen store and lactate accumulation in skeletal muscle f men during intense bicycl exercise Scand J Sport Sci Accepted for publication
- KOMI P V & KARLSSON J 1979 Physical performance skeletal muscle enzyme activity and fibre types in monozygous and dizygous twins f both sexes Acta Physiol Scand Suppl 462
- LAESSLE B W, KALLNER, L, RANLQVIST M L & CARLSON L A, 1971 Relation hip in man between plasma free f tty acids and myocardial metabolism of carbohydrate substrate Lancet 2 448-450
- LOWRY O H & PASSONNEAU J V 1973 A flexible y tam f enzymatic analysis New York Academic Press
- MORGAN H E, RANLQVIST P J & REGAN D M 1959 Regulation f glucose uptake by muscle J The effect f insulin, anoxia, ascorbic acid and 2,4-dinitrophenol on membrane transport and intracellular phosphorylation f glucose in the isolated heart Biochem J 73 573-582
- MORGAN H E & PARRINELLI A 1944 Regulation of glycogenolysis in muscle III Control f muscle glycogen phosphorylation activity J Biol Chem 239 2440-2445
- NEVILLER E A 1977 The regulation f extracellular and intracellular fuel supply during sustained exercise Annual of the New York Academy of Science 301 81-91
- NEVILLER E A & START C 1973 Regulation in metabolism John Wiley and Sons London
- NEVILLER, E.A & CHASTICE, B 1979 Theoretical principles in the pyrochlore control f metabolic pathways and their application to glycogenolysis in muscle J Mol Cell Cardiol 11 639-656
- OLSSON A G, EKLUND B, KALLNER, L & CARLSON L A 1975 Extraction f endogenous plasma triglycerides by the working human forearm muscle in the f t i g at c Scand J Clin Lab Invest 35 231 236
- OLSSON A G, EKLUND B, RICCARDI C, WALLDIUS G & KALLNER, L 1977 Free f ty acid and carbohydrate metabolism f the working forearm muscle in type IV hyperproteinemia Scand J Clin Invest 7:549-556
- PETERS P P & VAN ELYKE D D 1932 Quantitative clinical chemistry Vol II Tindall & Cox Baltimore
- RANDOLPH P J & SMITH G R 1958 Regulation of glucose uptake by muscle I The effect of insulin, anaerobiosis and 11 poisons on the uptake of glucose and release of potassium by isolated rat diaphragm Biochem J 70 490-500
- RENNER M J, WINDER, W W & HOLLOSZY J O 1978 A surprising effect f increased plasma free fatty acids on muscle and liver glycogen content in the exercising Biochem J 154 647-655



- SCHOLANDER P F 1947 Analyze of accurate estimation of respiratory gases in one half cubic centimeter sample J Biol Chem 167:235-250
- SEVERINGHAUSE J W & BRADLEY A F 1958 Electrode for blood  $pO_2$  and  $pCO_2$  determination J Appl Phys 11:3 515-520
- SUTHERLAND E W & EYE I & BUTCHER R W 1965 The action of epinephrine and the role of the adenylylase system in hormone action Rec Progr Horm Res 21:623-646





**ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 488**

**INTRACELLULAR LOCALIZATION OF  
CARBONIC ANHYDRASE IN SOME VERTEBRATE  
NEPHRONS**

**By**

**YVONNE RIDDERSTRÅLE**



**INTRACELLULAR LOCALIZATION OF  
CARBONIC ANHYDRASE IN SOME VERTEBRATE  
NEPHRONS**

**By**

**YVONNE RIDDERSTRÅLE**

**UPPSALA 1980**



**ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 488**

**FROM THE DEPARTMENT OF ANIMAL PHYSIOLOGY  
SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES  
S-760 07 UPPSALA 7 SWEDEN**

**INTRACELLULAR LOCALIZATION OF  
CARBONIC ANHYDRASE IN SOME VERTEBRATE  
NEPHRONS**

**By**

**YVONNE RIDDERSTRÅLE**





ACTA PHYSIOLOGICA SCANDINAVICA  
SUPPLEMENTUM 488

From The Department of Animal Physiology  
Swedish University of Agricultural Sciences  
S-750 07 Uppsala 7 Sweden

ABSTRACT

*KIDDERSTRÅLE Y Intracellular localisation of carbonic anhydrase  
in some vertebrate nephrons*

The Han son histochemical method for carbonic anhydrase has been modified by embedding of the tissue in a resin before sectioning and incubation. While retaining the now generally accepted validity and specificity of the original method it has several advantages. It is simple and rapid and appears to give unusually sharp localization of the stain. The section thickness may be varied to suit light microscopy and electron microscopy with resolution down to 25 Å. The method has been discussed specially with regard to false localization.

The intracellular distribution of carbonic anhydrase in the kidneys of frogs and birds has been examined.

As a basis for the histochemical investigation an ultrastructural analysis of the avian nephron has been performed using mainly young swifts. Thin tubules are found exclusively in medullary nephrons and their cells are of an unusual structure combining high cell thickness with strong development of the paracellular route. The avian initial collecting tubule contains a high proportion of dark cells containing mainly baso-lateral carbonic anhydrase. The distribution and morphology of dark cells in vertebrate nephrons has been surveyed. These cells are also compared with oxyntic cells and it is suggested that the dark cells are responsible for the terminal acidification of urine taking place in the kidney.

A simplified terminology for the vertebrate nephron is suggested.

**Key-words:** carbonic anhydrase nephron histochemistry electron microscopy acidification

Uppsala 1980  
ISSN 0302-2994

This thesis is based on the following papers:

Distribution of carbonic anhydrase in the frog nephron 1974  
Gudmar Lönnholm & Yvonne Ridderstråle *Acta physiol scand* 90 764-778

Intracellular localization of carbonic anhydrase in the frog  
nephron 1976 Yvonne Ridderstråle *Acta physiol scand* 98  
465-469

Intracellular distribution of carbonic anhydrase in the rat  
kidney 1980 Gudmar Lönnholm & Yvonne Ridderstråle  
*Kidney Int* 17:2 In press

Ultrastructure and localization of carbonic anhydrase in the  
avian nephron 1980 Yvonne Ridderstråle *Swedish J Agric Res*  
In press

The thesis contains a discussion of the histochemical methods  
used (with some new experimental data) a short survey of the  
histochemical results and a note of the terminology of the  
nephron

## INTRODUCTION

Following up an idea by Kurata (1953) Häusler (1958) developed the first useful histochemical method for carbonic anhydrase. His method involved the precipitation of cobalt compound at sites alkalized by the enzymatic dehydration of carbonic acid in sections floated on the surface of the incubation fluid. Mainly by adding phosphate to the incubation medium Hansson (1967) improved the method considerably and was able to show that the staining could be inhibited by concentrations of acetazolamide which were so low that only the specific inhibitory effect of the drug could be responsible for the effect. He also showed that the staining by his histochemical method agreed well with biochemical data and presented a series of pictures of impressive beauty showing the carbonic anhydrase distribution in several organs (Hansson 1968).

Various objections to Häusler's and Hansson's methods have been raised by Fandl et al (1959) and Churg (1973) but especially by Mutha (1972, 1977). The critique has been rebutted by Rosen et al (1972), Lönnholm (1974, 1980) and Maren (1980). The result of this discussion appears to be that Hansson's method (properly used) has emerged as a valid histochemical method with a high degree of specificity. This is the basis and starting point of the following discussion of some variations of the method and possible limitations of these.

## THE METHODS

The several variations of the method will be discussed mainly on the basis of the experience gained by Lönnholm & Riddström (1974, 1980) and Ridderstål (1976, 1980) and the discussion will be concentrated on the techniques used in order to adapt the method to electron microscopy.

These methods differ mainly with regard to the stage at which the incubation of the sections is performed. In the original Hanson method as used for electron microscopy by Yokota (1969) Cassidy & Lightfoot (1974) Rosen (1974) and Lönnnerholm & Ridderstrål (1974) and with Hsu's method by Hsu et al (1966) and Cross (1970) the incubation is performed with freeze-cut sections of glutaraldehyde fixed material. After washing and treatment with ammonium sulphide the sections are embedded in a suitable resin for electron microscopy and ultrathin sections are cut. At some stage counterstaining with osmium, lead or uranyl may be used.

The staining takes place in a multicompartiment system divided by lipid membranes more or less denatured and disrupted by fixation, freezing and sectioning. These membranes influence the diffusion not only of the components of the incubation fluid but also of the primary and final reaction products. The factors influencing the distribution of the ensuing precipitate are thus very difficult to penetrate. This is a drawback, however, which this method has in common with most histochemical methods (Pearse 1968). More important are certain practical considerations which have been discussed by Lönnnerholm & Ridderstrål (1974). One of the difficulties with this method is that in order to get a reasonably coherent picture the ultrathin sections are cut parallel to the surface of the original thick section. The precipitate is thickest at the upper surface and thins out successively downwards and disappears completely a few microns from the surface. The line dividing the stained from the unstained part of the section is fairly straight but the wavy. The distribution of the precipitate and its density in the thin sections are of course distinctly influenced by this circumstance (Lönnnerholm & Ridderstrål 1974 Fig. 4). The interpretation of the picture is thus to a certain degree subjective. In addition the staining of

for instance mitochondria was rather erratic. In a few cases the interior of the mitochondria was all stained except the membrane and in other cases the interior was completely unstained.

Some of the difficulties encountered appeared possible to overcome with suitable minor modifications. The erratic appearance of the precipitate on the other hand was considered more serious and prompted efforts aimed at the development of a method where the incubation takes place after embedding and sectioning for electron microscopy.

The obvious difficulty with this approach is that the enzymes may be completely inactivated by the embedding procedure or that the components of the incubation solution may be excluded from the sites of enzyme activity.

After extensive trials it was found that the resin JB-4 (Sorvall and Polysciences) appeared suitable. This is again produced by polymerization of a mixture of glycol methacrylate and polyethylene glycol (Ruddell 1967 b).

The use of this resin made possible the development of a method for light microscopy which was convenient and did not require any special apparatus. It resulted in excellent preservation of cell morphology and gave very sharp and highly reproducible localization of the stain. Thin sections (down to 0.5  $\mu$ m) resulted in sharp pictures and resolution to the limit of light microscopy.

Though the resin is primarily intended for light microscopy suitable precautions make it possible to cut sections thin enough for electron microscopy (Ridd et al. 1976, 1980).

When this method is used the lipid membranes are dissolved during the embedding and the section incubated essentially as a one-compartment system through which the component of the incubation medium and primary and final reaction products can diffuse. The diffusion rates are undoubtedly considerably lower than in water. The water occupies about 30 - 40 per cent of the water.

soaked resin. Thus the diffusion rates of small water soluble molecules and ions would be expected to be lowered about 15-30 times (Meares 1968). The diffusion of larger molecules and especially crystallization nuclei would of course be much more hampered (White et al 1961).

The method also has its weaknesses and limitations. Some of these will be detailed and discussed below.

The difficulties are most obvious with regard to the method for electron microscopy. The reason for this is probably that it is difficult to standardize sufficiently the washing after the incubation. The washing fluid undoubtedly dissolves a minute amount of the precipitate and this of course is especially noticeable in the areas of the section which are least stained. This is the reason why washing time is reduced compared to the original method. This difficulty becomes much more noticeable when sections around 0.1  $\mu$ m or thinner are used. In order to obtain highly reproducible results with regard to the depth of staining the section thickness should not be less than about 0.2  $\mu$ m.

As the section thickness is thus rather high it is essential to use a high acceleration voltage in the electron microscope. In most cases 100 kV has been used but the resolution is noticeably improved by the use of 200 kV.

The lack of reproducibility is limited to the above mentioned variability of depth of staining and sometimes lack of staining in the thinnest sections in areas where thicker sections show staining.

## FALSE LOCALIZATION

When the Hansson method is used mainly to examine the cellular distribution of enzymes the question of specificity is the most important one. When the aim of the work is to study the intracellular distribution and the question of specificity is satisfactorily answered the possibility of false localization of the stain comes to the foreground. The general question of false localization in histochemistry has been much discussed. The terminology and general concepts used by Holt et al. (1958) and Ilit (1959), Pear (1968) and especially van Duijn (1974) are used in the following discussion.

Ridderstrål (1976) discussed the question of false localization of stain to cell membranes in the frog kidney. If membranes contain preformed crystallization nuclei it seems natural to expect that these should be equally distributed all over the membrane. However, in one cell type the stain is localized at the apical-lateral cell membrane and in another cell type only the basolateral membrane is stained. This argues against the staining being due to false localization. This line of reasoning is of course equally applicable with regard to the results gained with avian and mammalian kidney.

However, another type of evidence appears possible. According to Hansson (1968) the staining is due to precipitation of a Co-compound as a result of local alkalinization at the enzyme site. When the incubation fluid is left in contact with the air for some time the loss of carbon dioxide leads to a general alkalization of the perfused layer of the fluid. This becomes visible by the formation of a surface film of precipitate. It appears reasonable to assume that the stain and the surface film are due to oversaturation with the same Co-compound. It should then be possible to demonstrate the distribution of preformed crystalliza-



tion nuclei in sections by incubating them on such supersaturated solutions

Sections were floated on the surface of an acetazolamide solution for 45 minutes and then transferred to incubation medium containing acetazolamide. Sections were picked up at various times and further processed. Up to about 15 minutes such sections showed very faint diffuse staining. At 20 minutes and especially at 30 minutes (when a surface film was clearly visible) a pronounced staining is observed. In the rat kidney the stain is composed of a weak diffuse stain about equally strong all through the cytoplasm and a strong nuclear stain which apparently equally affects all nuclei in the sections. In the proximal tubule the brush border shows clear but much weaker staining. In the pigeon the picture is similar. Of the staining appearing after prolonged incubation of inhibited sections only the staining of the brush border in the rat is at all similar to the staining occurring after short incubation times without inhibitor.

Clearly whenever a cell contains histochemically detectable carbonic anhydrase the nucleus may become stained whether the nucleus itself contains enzyme or not. This must especially be so in the case of the original method. After embedding in resin and with thin sections the possibility of long range diffusion should be much less.

Wistrand & Kinn (1977) and Wist and (1979) have shown that there is a separate membrane-bound isoenzyme in the kidney. This of course is excellent evidence that the membrane associated stain is due at least partly to enzymatic activity at this very site.

## ULTRASTRUCTURE OF THE PRECIPITATE AND RESOLUTION OF THE METHOD

With increasing incubation time the precipitate rapidly becomes very dense and then no finer structure can usually be observed in it with the electron microscope. In the early stage of incubation the membrane staining is however not only very thin but is also distinctly patchy. Sometimes ring-like structures usually with a diameter of about 400 - 500 Å are observed. Such structures have been seen in the membranes of the thick tubules of the nephron in the brew *Soximus* in the membranes covering microvilli in the colon of the rabbit (Figs 5, 6) and in the mammary gland of the rat. Occasionally the staining is thin enough to allow the fine structure of such rings to be observed. They are composed of a number of small dots (Fig. 6).

It does not appear possible to decide if these ring-like structures are artifacts of the method or possibly the result of preformed crystallization sites or if they give true pictures of the arrangement of the enzyme molecules. In any case they are related to the high resolution possible with the method. In Fig. 6 the resolution is about 25 Å. It would appear that the method may give an unusually sharp localization. The reason for this is probably the thinness of the sections incubated and the reduced mobility in the resin of the involved ions and crystal nuclei.

Despite the sharpness of localization it is usually impossible to decide whether a thin precipitate lies on the inside or the outside of a cell membrane. Occasionally when there is stain on both sides of a cell membrane this can be observed with certainty (Fig. 4).

## Enhancement of membrane staining

One of the main results of the investigation of kidneys with the present method is that the staining is to a very considerable extent localized to or closely associated with cell membranes. Only in a few cell types does cytoplasmic staining reach an intensity resembling that normally occurring at most stained membrane. This circumstance may appear to be at variance with the circumstance that the major part of renal carbonic anhydrase is cytoplasmic.

This problem was discussed by Ridderstråle (1976) who gave several possible explanations. One is that the method may be too insensitive to show any staining by a large amount of cytoplasmic enzyme distributed in a large volume whereas a small amount of membrane-associated activity in a small volume could reach a concentration high enough to give strong staining. Other possible explanations considered were differential inactivation of membrane-associated and cytoplasmic enzymes during fixation or embedding. For such explanations there appeared at that time to be little foundation.

With the more extensive knowledge of the results of the method now available and especially after the discovery of a membrane-bound isoenzyme of carbonic anhydrase in the kidney (Wistrand et al. 1977 and Wist and 1979) it appears possible to discuss the problem more thoroughly.

A very rough comparison of the enzyme concentrations in membranes with those of the cytoplasm may be made in mammalian kidneys. Wolling et al. (1975) give the surface of membranes in the rabbit proximal tubule as  $8 \mu\text{m}^2 / \mu\text{m}^3$ . The proximal tubule undoubtedly has much higher surface to volume ratio than

other renal cells thus this ratio may perhaps be taken as  $5 \mu\text{m}^2 / \mu\text{m}^3$  for the entire kidney. Assuming the membrane thickness to be 30 - 100 Å the ratio membrane volume / cell volume would be 1/20 - 1/70. The enzyme activity in membranes and mitochondria is about 5 per cent of total activity according to Witt and (1979). Thus the enzyme activity would probably be only slightly higher in membranes than in cytoplasm.

It then appears reasonable to look at other possibilities of explaining the usually much higher density of stain associated with membranes than with cytoplasmic areas.

One factor that may contribute is differential distribution of activity during fixation. It appears quite possible that enzyme molecules embedded in lipid could be less affected than the cytoplasmic enzyme.

The passage of the fixed tissue through increasing concentrations of monomers and perhaps especially the short heating in the polymerising mixture could lead to differential inactivation. In this case also it appears reasonable that the cytoplasmic enzyme could be more affected than the membrane-bound.

That inactivation of enzyme probably occurs during embedding is clear from a comparison of the results of Lönnholm & Riddström (1974) and Ridderström (1976). At equal times of incubation the original Hanson method gave much larger total amount of precipitate than the method with resin embedding. It is improbable that this considerable difference can be explained by the other factors which are different between the two procedures.

Provided the enzyme activity is higher in the membranes than in the cytoplasm the circumstances which may contribute substantially to give an impression of predominance of membrane staining.

The stained membranes often show distinct tendency to get thicker the longer the incubation time and in some instances fairly broad areas in the vicinity of stained membranes get

stained. This is seen very clearly in Figs 3 & 4 of Ridderstråle (1976). This phenomenon may of course simply be the result of diffusion of primary and final reaction products from the site of high enzyme activity in the membrane. It appears however that it may be the result of the additive effect of such diffusion and the products of cytoplasmic activity though the latter is in itself insufficient to result in staining. Thus cytoplasmic enzyme activity may contribute to the impression of predominant membrane staining. This effect would be expected to increase in importance the thicker the section.

Clearly there are several mechanisms which may give the result that cytoplasmic staining is considerably weaker than what corresponds to the levels of cytoplasmic enzyme. If this is true it represents a weakness of the method. At the same time this weakness may be a necessary prerequisite for the sharp and clear demonstration of membrane-bound enzyme which is the type of enzyme most likely to show an interesting intracellular distribution.

In any case this admittedly hypothetical discussion may serve to show that the method with incubation after embedding may for some purposes be less suitable than the original Hansson method and that sometimes a combination of methods is needed.

## Survey of carbonic anhydrase distribution in the kidneys of frog, swift and rat

The distribution of carbonic anhydrase according to the result of Riddström (1976-1980) and Lönnholm & Ridderstråle (1980) can best be condensed in schematic pictures shown in Fig. 1. For terminology see pages 17-18.

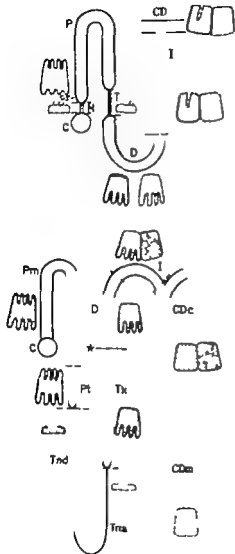
### *Fig. 1 Short remarks and explanations of the symbols*

Frog Occasionally the brush border in the proximal tubule shows activity. The distal tubule contains a segment with intense membrane staining especially its luminal membranes. The rest of the distal tubule is unstained and the exact location of the stained segment is not known.

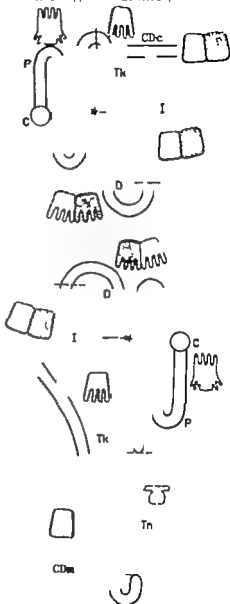
Bird The brush border of a minor part of the proximal tubules shows weak activity. In the thick tubule the lateral cell membranes are stained and in cortical nephrons also the apical cell membrane. The distal tubule contains the most heavily stained cells in the avian nephron with intense activity in the luminal membranes. Cytoplasmic activity is also found here. In the rest of the nephron the staining of the base-lateral membranes predominates.

Rat The dark cells in the initial collecting tubule and cortical duct show the strongest cytoplasmic staining but also the main segment shows considerable cytoplasmic activity. The strongest membrane-bound activity is found in the thick tubule and main segment.

**FROG**



## AVIAN CORTICAL NEPHRON

RAT

## AVIAN MEDULLARY NEPHRON

Capsule C	Initial collecting tubule I
Neck N	Collecting duct CD
Proximal tubule P	cortical duct CDc
main segment Pm	medullary duct CDm
terminal segment Pt	
Thin tubule Tn	— no membrane staining
descending segment Tnd	— weak membrane staining
ascending segment Tna	— strong membrane staining
Thick tubule Tk	: weak cytoplasmic staining
Macula densa *	: : strong cytoplasmic staining
Distal tubule D	

#### NOTE ON THE TERMINOLOGY OF THE NEPHRON AND THE DUCT SYSTEM

In the paper forming the basis of this there is several different systems of designating the parts of the nephron have been used. In Ridd (1980) and in the present paper the terminology deviates in some respects from what may appear most common in this rather confusing field. It appears appropriate at this point to explain very shortly the point of view which are at the basis of this nomenclature.

The overwhelming majority of renal research work deal with the mammalian kidney. Thus any system of terminology should be conveniently applicable to that nephron and should be based on morphological features as these are most universally ascertainable.

The nephron is supposed to end at the point where it joins another nephron or collecting duct.

The major divisions of the nephron are designated tubule the divisions of tubules are designated segment. The divisions of the collecting system are designated duct.



Whenever possible any term should be composed of two words only or if necessary for clarity at most three words are used

The designations of the segments are chosen so that the segment can be unequivocally described without reference to the tubule it is a part of

When applied to the mammalian kidney the system works out as shown in Fig. 2

Fig. 2

Capsule C

(Hook H)

Proximal tubule P

main segment Pa

terminal segment Pt

Thin tubule Tn

descending segment Tnd

ascending segment Tna

Thick tubule Tk

medullary segment Tkm

cortical segment Tko

macula densa

(intercalated segment Tki)

Distal tubule D

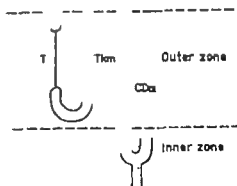
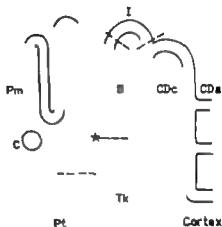
Initial collecting tubule I

Collecting duct CD

ascending duct CDa

cortical duct CDc

medullary duct CDm.



It perhaps needs emphasizing that the transitional segment is not equivalent to the straight part of the proximal tubule. The boundary between the convolutions and the straight part does usually not coincide with the change in cytology which is the boundary between main segment and transitional segment.

In most cases there is no intercalated segment. When there is one it is perhaps better included in the thick tubule on the basis of its cytology (Kaisling et al. 1977).

The neck is dilated part of the nephron occurring in lower vertebrates where the thin tubule is also dilated.

#### ACKNOWLEDGMENTS

I am deeply indebted to my teacher Professor Ivar Spärber for introducing me to the Great Adventure of research work. His enthusiasm and patience have been of great importance especially when things went wrong.

The interest and valuable help of Professor F. Wistrand and Professor P.-G. Knutsson is gratefully acknowledged.

The support and skilful work of Emilia Fuch, Ulla Karlsson and Ingrid Wennberg have been invaluable.

For careful scrutiny of manuscripts and proof I am much indebted to Dr Christina Sperbe.

I am also much obliged to my comrades (past and present) of our department for their educating, truth-telling and good fellowship.

An electron microscope with high accelerating voltage has been available through the courtesy of Professor O. Vingebo and Ingemar K. Kalm.

The investigations have been supported by grant 3285-1 from the Swedish Natural Science Research Council and grant from the Swedish University of Agricultural Sciences.

## REFERENCES

- Cassidy M M & Lightfoot F O 1974 Carbonic anhydrase In: Electron microscopy of enzymes Principles and methods (ed M A Hayat) Vol 3 77-108 van Nostrand Reinhold Comp New York
- Churg A. 1973 Carbonic anhydrase histochemistry: Evidence for non-enzymatic reaction and artifact production *Histochemia* 36 293-302
- Cross E A.M 1970 Ultrastructural localisation of carbonic anhydrase in rat stomach parietal cells *Hist chemia* 22 219-225
- van Duijn P 1974 Fundamental aspects of enzyme cytochemistry In: Electron microscopy and cytochemistry (ed Wisse E Daems W Th Molenaar I & van Duijn P) 3-23 North-Holland Publishing Comp Amsterdam
- Fand S B Levin H J & Erwin H L 1959 A reappraisal of the histochemical method for carbonic anhydrase *J Histochem Cytochem* 7 27-33
- Hansson H P J 1967 Histochemical demonstration of carbonic anhydrase activity *Histochemia* 11 112-120
- Hansson H P J 1968 Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport *Acta physiol scand* 73 427-434
- Holt S J & O'Sullivan D G 1958 Studies in enzyme cytochemistry I Principles of cytochemical staining methods *Roy Soc Proc Ser B* 148 465-480
- Holt S J 1959 Factors governing the validity of methods for enzymes and their bearing upon the cyphosphatase technique *Exp Cell Res Suppl* 7 1-
- Hopsu V Arstila A.U & Helminen H 1966 Studies in electron microscopy of arylsulphatase and carbonic anhydrase *J Histochem Cytochem* 14 762

- Huusle G 1958 Zur Technik und Spezifität der histochemischen Carboanhydrasenachweise im Modellsversuch und in Gewebsschnitten von Rattenmilchen *Histochemie* 1 20-47
- Kaisling B P & Krix W 1977 The transition of the thick ascending limb of Henle loop into the distal convoluted tubule in the nephron of the rat kidney *Cell Tissue Res* 182 111-118
- Kurata Y 1953 Histochemical demonstration of carbonic anhydrase activity *Stain Techn* 28 231-233
- Lönnerholm G 1974 Carbonic anhydrase histochemistry: a critical study of Hansson's cobalt-phosphate method *Acta physiol scand Suppl* 418
- Lönnerholm G & Riddström Y 1974 Distribution of carbonic anhydrase in the frog nephron *Acta physiol scand* 90 764-778
- Lönnerholm G 1980 Carbonic anhydrase in skeletal liver and rabbit skeletal muscle: further evidence for the specificity of the histochemical cobalt-phosphate method of Hansson *J Histochem Cytochem* In press
- Lönnerholm G & Riddström Y 1980 Intracellular distribution of carbonic anhydrase in the rat kidney *Kidney Int* 17 2
- Maren TH 1980 Kinetics, equilibrium and inhibition in the Hansson histochemical procedure for carbonic anhydrase: A validation of the method *Histochem J* In press
- Meares P 1968 Transport in ion-exchange polymers *Int Diffusion in polymers* (ed Crank J & Park GS) 403-428 Academic Press London & New York
- Muth TF 1972 A critical evaluation of the histochemical methods for carbonic anhydrase *J Histochem Cytochem* 20 319-330
- Muthe TF 1977 On the lack of specificity of the cobalt-bicarbonate method for carbonic anhydrase *J Histochem Cytochem* 25 1043-1050

- Pearse A G R. 1968 Histochemistry 3d ed Vol 1 Churchill  
London
- Ridderstråle Y 1976 Intracellular localization of carbonic  
anhydrase in the frog nephron Acta physiol scand 98 465-469
- Ridderstråle Y 1980 Ultrastructure and localization of carbonic  
anhydrase in the avian nephron Swedish J Agric Res In press
- Rosen S & Musser G L 1972 Observations on the specificity of  
newer histochemical methods for the demonstration of carbonic  
anhydrase activity J Histochem Cytochem 20 951-954
- Rosen S 1974 Carbonic anhydrase - alternativ method In:  
Electron microscopy of enzymes Principles and methods (ed M A  
Hayat) Vol 3 109-134 van Nostrand Reinhold Comp New York
- Ruddell C L 1967a Hydroxyethyl methacrylate combined with poly-  
ethylene glycol 400 and water an embedding medium for routine  
1 - 2 micron sectioning Stain Techn 42 119-123
- Ruddell C L 1967b Embedding media for 1 - 2 micron sectioning  
2 Hydroxyethyl methacrylate combined with 2 butoxyethanol  
Stain Techn 42 253-255
- Welling L W & Welling D J 1975 Surface areas of brush border  
and lateral cell walls in the rabbit proximal nephron Kidney  
Int 8 343-348
- White M L & Dorion G H 1961 Diffusion in a crosslinked acryl-  
amid polymer gel J Polym Sci 55 731-740
- Wistrand E J & Kinne R 1977 Carbonic anhydrase activity of  
isolated brush border and basal-lateral membranes of renal  
tubular cells Pflügers Arch 370 121-126
- Wistrand E J 1979 Renal membrane-bound carbonic anhydrase  
Purification and properties Upsala J Med Sci suppl 26 75
- Yokota S 1969 Electron microscopic demonstration of carbonic  
anhydrase activity in mouse liver cells Histochem 19 255-  
261



Fig 3 Section from cortex of rat kidney. Inhibited by acetanilide incubated 30 min. Strong unspecific staining of nuclei weaker of brush border. 640 X

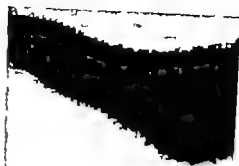


Fig 4 Erythrocyte of *Sorex araneus* showing carbonic anhydrase activity on both sides of the cell membrane. 18 800 X

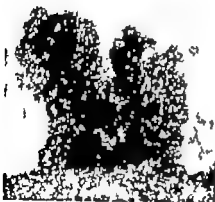


Fig 5 Microvilli from surface epithelium of rabbit colon showing ring-like staining pattern. 34 000 X

Fig 6 Similar to Fig 5. 124 000 X







## REFERENCES

- Cassidy M M & Lightfoot F W 1974 Carbonic anhydrase In: Electron microscopy of enzymes Principles and methods (ed M A Hayat) Vol 3 77-108 van Nostrand Reinhold Comp New York
- Churg A 1973 Carbonic anhydrase histochemistry: Evidence for non-enzymatic reaction and artifact production Histochemie 36 293-302
- Cross S A M 1970 Ultra structural localisation of carbonic anhydrase in rat stomach parietal cells Histochemie 22 219-225
- van Duijn P 1974 Fundamental aspects of enzyme cytochemistry In: Electron microscopy and cytochemistry (ed Wisse E Daems W Th Molenaar I & van Duijn P) 3-23 North-Holland Publishing Comp Amsterdam
- Fand S B Levine B J & Erwin H L 1959 A reappraisal of the histochemical method for carbonic anhydrase J Histochem Cytochem. 7 27-33
- Hansson H P J 1967 Histochemical demonstration of carbonic anhydrase activity Histochemie 11 112-128
- Hansson H P J 1968 Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport Acta physiol scand 73 427-434
- Holt S J & O Sullivan D G 1958 Studies in enzyme cytochemistry I Principles of cytochemical staining methods Roy Soc Proc Ser B 148 465-480
- Holt S J 1959 Factors governing the validity of staining methods for enzymes and their bearing upon the Gomori acid phosphatase technique Exp Cell Res Suppl 7 1-27
- Hopsu V Arstila A U & Helminen H 1966 Studies on the electron microscopy of arylsulphatase and carbonic anhydrase activities J Histochem Cytochem 14 762

- Häusler G 1958 Zur Technik und Spezifität des histochemischen Carboanhydrasenachweises im Modellversuch und in Gewebsschnitten von Rattenmilien. *Histochem* 1 20-47
- Kal ling B Pate S & Kris W 1977 The transition of the thick ascending limb of Henle's loop into the distal convoluted tubule in the nephron of the rat kidney *Cell Tiss Res* 182 111-118
- Kurata Y 1953 Histochemical demonstration of carbonic anhydrase activity *Stain Techn* 28 231-233
- Lönnholm G 1974 Carbonic anhydrase histochemistry: a critical study of Hansson's cobalt-phosphate method *Acta physiol scand Suppl* 418
- Lönnholm G & Ridderstråle Y 1974 Distribution of carbonic anhydrase in the frog nephron *Acta physiol scand* 90 764-778
- Lönnholm G 1980 Carbonic anhydrase in rat liver and rabbit skeletal muscle: further evidence for the specificity of the histochemical cobalt-phosphate method of Hansson. *J Histochem Cytochem* In press
- Lönnholm G & Ridderstråle Y 1980 Intracellular distribution of carbonic anhydrase in the rat kidney *Kidney Int* 17:2
- Maren T H 1980 Kinetic equilibrium and inhibition in the Hansson histochemical procedure for carbonic anhydrase: A validation of the method *Histochem. J* In press
- Meares P 1968 Transport in ion-exchange polymers. In: *Diffusion in polymers* (ed Crank J & Park G S) 403-428 Academic Press London & New York
- Muth TF 1972 A critical evaluation of the histochemical methods for carbonic anhydrase. *J Histochem Cytochem* 20 319-330
- Muther T F 1977 On the lack of specificity of the cobalt-borbonate method for carbonic anhydrase. *J Histochem Cytochem* 25 1043-1050

- Pearse A.G.R. 1968 *Histochemistry* 3d ed Vol 1 Churchill  
London
- Ridderstråle Y 1976 Intracellular localization of carbonic  
anhydrase in the frog nephron *Acta physiol scand* 98 465-469
- Ridderstråle Y 1980 Ultrastructure and localization of carbonic  
anhydrase in the avian nephron *Swedish J agric Res* In press
- Rosen S & Musser G.L. 1972 Observations on the specificity of  
new histochemical methods for the demonstration of carbonic  
anhydrase activity *J Histochem Cytochem* 20 951-954
- Rosen S 1974 Carbonic anhydrase - alternative method In  
*Electron microscopy of enzymes Principle and methods* (ed M A  
Hayat) Vol 3 109-134 van Nostrand Reinhold Comp New York
- Ruddell C.L. 1967a Hydroxyethyl methacrylate combined with poly-  
ethylene glycol 400 and water; an embedding medium for routine  
1 - 2 micron sectioning *Stain Techn* 42 119-123
- Ruddell C.L. 1967b Embedding media for 1 - 2 micron sectioning  
2 Hydroxyethyl methacrylate combined with 2-butoxyethanol  
*Stain Techn* 4 253-255
- Welling L.W. & Welling D.J. 1975 Surface areas of brush border  
and lateral cell walls in the rabbit proximal nephron *Kidney  
Int* 8 343-348
- White M.L. & Dion G.H. 1961 Diffusion in a crosslinked acryl-  
amid polymer gel *J Polymer Sci* 55 731-740
- Wistrand P.J. & Kinne R. 1977 Carbonic anhydrase activity of  
isolated brush border and basal-lateral membranes of renal  
tubular cell *Pflügers Arch* 370 121-126
- Wistrand P.J. 1979 Renal membrane-bound carbonic anhydrase  
Purification and properties *Uppsala J Med Sci Suppl* 26 75
- Yokota S 1969 Electron microscopic demonstration of carbonic  
anhydrase activity in mouse liver cells *Histochemie* 19 255-  
261



Fig 3 Section from cortex of rat kidney. Inhibited by acetazolamide incubated 30 min. Strong unspecific staining of nuclei weaker of brush border 640 X



Fig 4 Erythrocytes of *Sorbus amarus* showing carbonic anhydrase activity on both sides of the cell membrane 18 600 X



Fig 5 Microvilli from surface epithelium of rabbit colon showing ring-like staining pattern. 34 000 X

Fig 6 Similar to Fig 5 124 000 X



Indirect support according to Dreyfus (1976) for the view that the symptoms of thiamine deficiency are not the result of an impaired coenzyme function of Th DP is that the deficiency symptoms are much easier precipitated if the thiamine deficiency is combined with a large carbohydrate load. The effect of the carbohydrate load was considered to be due to stimulation of the enzyme systems where Th DP is a coenzyme at the expense of another thiamine-requiring mechanism that is responsible for the deficiency symptoms.

A possible function of thiamine in neuromuscular transmission was investigated in the present work by studying

- 1 the formation of the methylthiamine-like substance (MTLS) from choline and thiamine
- 2 the formation of the MTLS after nerve stimulation
- 3 the release of radioactive thiamine from the stimulated phrenic nerve-diaphragm preparation
- 4 the binding and effect of thiamine on nicotinic acetylcholine receptors and
- 5 the effect of thiamine on neuromuscular transmission in rat masseter muscle

## COMMENTS ON METHODS

The different methods have been described in detail in the individual papers and therefore the methodological principles are only shortly summarized here

In paper I and II the methods for the extraction of the HTLS are given. The HTLS was extracted from homogenized cat ventral roots using a counter ion dissolved in an organic solvent. The homogenization was carried out in an organic solvent which has two advantages: immediate denaturation of the proteins and a partial solubilization of the lipids. These facts may explain why the HTLS was not found in earlier studies where choline metabolites have been extracted first after acid precipitation. Accordingly, when the ventral roots in the present study were homogenized in trichloroacetic acid, no HTLS was recovered from the supernatant after centrifugation. The occurrence of HTLS in the organic homogenization medium was considered improbable since only trace amounts of HTLS were found if radioactive line was added to nonradioactive ventral roots before the homogenization.

In paper V the binding of thiamine to nicotinic acetylcholine receptors was studied both by using an isolated receptor protein from the electric organ of Torpedo marmorata and by using end plate receptors from frog sartorius muscle. The isolated nicotinic acetylcholine receptor has certain characteristics in common with nicotinic receptors in the frog muscle end plate: it binds nicotinic antagonists in low concentrations. However, the binding of acetylcholine to the isolated nicotinic acetylcholine receptor requires a high acetylcholine concentration ( $10^{-6}$  M) whereas the



acetylcholine concentration that has an effect on the frog end-plate is much lower ( $10^{-9}$  M). Since differences in binding characteristics between the two types of receptors exist it is important to compare results achieved with the isolated receptor with results from frog muscle end plate receptors. If the binding of a substance to nicotinic receptors is studied by using both techniques and the results are the same then the probability is increased that the binding of this substance to nicotinic acetylcholine receptors or to structures closely related to their function (ionophores) is studied.

In paper VI the effect of thiamine on post-tetanic potentiation (PTP) was studied using the masseter muscle of the rat. The masseteric nerve was stimulated with supramaximal impulses the voltage of the stimulation impulses being 150 % of that which yielded maximal muscle twitches. Therefore the observed changes in muscle tension of the muscle contractions do not depend on differences in the recruitment of muscle fibres. In some experiments field stimulation of the masseter muscle was made. No difference in results between stimulation of the masseteric nerve and field stimulation was noted. The pre tetanic and post-tetanic stimulation frequency was 0.2 Hz and the impulse duration 0.2 ms. When the low frequency stimulation is interrupted by a short tetanus a transitory increase in the peak tension of the isometric muscle twitch response (PTP) is seen in fast mammalian muscle. PTP in fast mammalian muscle has been described to be due to an increased contractility of the muscle fibres (Standaert 1964).

## RESULTS AND DISCUSSION

### Chromatographical characterization of the MTLs

When the radioactive  $^3\text{H}$ -choline was injected into the ventral horn nuclei in the spinal cord of the cat radioactivity appeared in the ventral roots after less than 30 min (paper III). Part of the radioactivity was extracted by homogenizing the roots in allylcyanide containing sodium-tetraphenylboron (counter ion extraction). Compounds which contain a quaternary ammonium ion as the single charge are thereby bound to the sodium-tetraphenylboron and extracted into the organic phase (Fonnum 1969). By adding a strong acid to the organic phase (e.g.  $\text{HCl}$ ) the quaternary ammonium compounds are regained into the inorganic phase which thereafter can be freeze-dried.

When the freeze dried extracts from the radioactive ventral roots were dissolved in ethanol and submitted to paper chromatography then three peaks of radioactivity could be found. Two peaks had as expected the chromatographical characteristics of acetylcholine and choline but the third one could not be identified as any known choline metabolite. Thiamine was found to be the only substance with chromatographical properties similar to those of the unknown substance. Since the methyl groups of the quaternary ammonium ion of choline were labelled the possibility was considered that the unknown compound was identical with methylthiamine formed by methylation.

Methylthiamine was synthesized as described in paper III and chromatographed together with the unknown compound in

several chromatographical systems

In all chromatographical systems tried the unknown compound behaved as methylthiamine. Radioactive thiamine was injected into the ventral horn nuclei. Also in this case a radioactive peak could be found in the chromatograms of ventral root extracts. Thus a compound (methylthiamine-like substance (MTLS)) with the same chromatographical characteristics as methylthiamine may have been formed from both choline and thiamine. The evidence for identity between the MTLS and methylthiamine is 1. chromatographical identity between the MTLS and methylthiamine in several chromatographical systems and 2. the MTLS is formed both from choline and thiamine. Such evidence for identity between two compounds is only indirect and therefore the substance is called MTLS.

#### MTLS and choline metabolism

The MTLS was formed in the ventral roots after the injection of radioactive choline. It was considered to be of interest to study whether the MTLS was formed from choline itself or from any of its metabolites (paper : III). Choline is metabolized in three directions 1. to acetylcholine 2. to phospholipids and 3. to betaine.

Radioactive betaine did not serve as a precursor for the MTLS when injected into the ventral horn nuclei. Therefore it was considered improbable that the MTLS was formed after demethylation of betaine or from any of its metabolites. To study a possible relationship between the formation of the MTLS and phospholipids the time course for formation of the MTLS and phospholipids was compared. The formation of

the MTLs was maximal after two hours. Thus the formation of the MTLs does not seem to be dependent on the formation of phospholipids. When the amounts of acetylcholine and MTLs in ventral and dorsal roots were compared, the amounts of both acetylcholine and MTLs were higher in ventral roots than in the dorsal roots. When the time course of the formation of acetylcholine and MTLs in ventral roots was studied, it was found that both compounds reached their maximal level in about 30 min. Thus it seems as if the formation of acetylcholine and of MTLs are related. The immediate precursor for the MTLs can according to these studies be either choline or acetylcholine. The biological importance however of the formation of the MTLs was unclear. The idea was put forward that the formation of methyithiamine could reflect the activation of nicotinic receptors.

The formation of the MTLs following administration of  $^3\text{H}$  choline did not occur in liver, kidney or spleen and may therefore be specific for nerve tissue and especially motor nerves. Since it is not formed in all tissues it is improbable that the formation of the MTLs only represents a common break-down product of choline or thiamine.

Formation of the MTLs in the stimulated rat hemidiaphragm. To investigate whether the formation of the MTLs could be influenced by changes in nerve activity (as was the formation of compound X in v. Muralts study) the formation of the MTLs in the stimulated and nonstimulated phrenic nerve-diaphragm preparation was studied. It was reported in paper IV that the amounts of MTLs formed were found to be higher in stimulated than in unstimulated rat diaphragms. The formation of the MTLs was inhibited by d-tubocurarine (12 mg/kg). The MTLs could only be extracted from the nerve

terminal region of the diaphragm muscle (can easily be seen as a band along the muscle) Thus the level of MTLs in the innervated part of the rat diaphragm increased as a result of nerve stimulation as did compound X in v Murali's study (1958) In addition the formation of MTLs was inhibited by curare suggesting that the formation of the MTLs is linked to the activation of curare-sensitive receptors

Release of  $^{35}\text{S}$ -thiamine from the stimulated rat diaphragm  
When the phrenic nerve of the hemidiaphragm was stimulated a release of radioactive thiamine into the bath was found in the presence of d-tubocurarine (paper IV) Since the muscle contractions were blocked by d-tubocurarine the thiamine in the bath after nerve stimulation probably originated from the nerve fibres Thiamine is mainly stored as phosphates Therefore a dephosphorylation of thiamine or an increase in extractable non-phosphorylated thiamine seems to occur during nerve stimulation In electrically stimulated frog sciatic nerves v Murali (1947) found an increase in thiamine extracted by Ringer solutions results which showed that the amount of free (=extractable) thiamine had increased as a result of the stimulation A dephosphorylation of thiamine phosphates was noted by Itokawa and Cooper (1970) who found an increase in non-phosphorylated thiamine in the perfusion fluid from rat sciatic nerve or rat spinal cord when neuroactive agents such as acetylcholine, tetrodotoxin, ouabain or LSD were added to the perfusion fluid Thus electrical stimulation or addition of certain neuroactive agents to nervous tissue might induce a shift from bound to free thiamine and/or a dephosphorylation of the thiamine phosphates

Binding of thiamine to nicotinic acetylcholine receptors  
Since the increase in the MTLs in the phrenic nerve-diaphragm preparation was blocked by d-tubocurarine it was considered to be of interest to study a possible binding to and effect of thiamine on nicotinic acetylcholine receptors. Nicotinic acetylcholine receptors can be isolated and purified from electric organs of fish (Eldefrawi and Eldefrawi 1973 Heilbronn et al 1974 Klett et al 1973 Meunier et al 1974) Such material was considered to be suitable for binding studies. In paper V it was shown that thiamine binds reversibly to the isolated nicotinic acetylcholine receptor protein ( $K_D=10^{-5}$  M). The binding of thiamine was inhibited by the venom toxin from Naja naja siamensis which is a specific antagonist for postsynaptic nicotinic receptors (Chang and Lee 1966). The binding of thiamine to the isolated nicotinic receptor was not inhibited by acetylcholine.

A possible effect of thiamine on post-synaptic nicotinic acetylcholine receptors was investigated by studying the miniature end plate potentials of the frog muscle end plate (paper V). The miniature end plate potentials are the result of nicotinic acetylcholine receptor activation due to release of acetylcholine. When thiamine ( $10^{-5}$  M) was applied to the end plate preparation the amplitude of the miniature end plate potentials decreased reversibly and significantly. No effect on the resting membrane potential was noted. The decrease in the miniature end plate potentials induced by thiamine indicated that thiamine blocks the action of acetylcholine. A decrease in the miniature end plate potentials by thiamine can be induced by a block of the sodium channels in the end plate that give rise to the miniature end plate potentials registered. Since the binding of acetylcholine

was not inhibited by thiamine in the isolated receptor from Torpedo marmorata these results could indicate that thiamine binds to the sodium channels and not to the acetylcholine binding site

#### **Pyridoxamine and neuromuscular transmission**

In paper VI the effect of thiamine on neurotransmission at higher stimulation frequencies ( $> 1\text{ Hz}$ ) was studied in rat masseter muscle. It is a typical fast muscle since it responds with individual twitches up to frequencies of 30 Hz. Since the masseter muscle is fast the tension of the individual muscle twitches will increase at high stimulation frequencies (stair case effect) and a frequency-dependent increase in contractility (PTP) will develop after moderate tetanic stimulations.

The effect of thiamine on the development of PTP in the untreated masseter muscle was investigated by administering thiamine intravenously. No effect was noted on the PTP or on the ordinary single twitches. When the effect of pyridoxamine and thiaminase-containing preparations (fern extract) on the PTP was studied then the muscle twitches at stimulation frequencies of  $> 1\text{ Hz}$  were decreased and PTP was abolished. Intravenous injections of thiamine alone there after restored the PTP and the muscle twitches at high frequency stimulation. Oxithiamine had no effect on the muscle twitches induced by high frequency stimulation or on the PTP. No effect on directly excited muscle twitches was seen after pyridoxamine administration. Therefore the effects of pyridoxamine and thiamine are on the nerve and not on the muscle.

Pyridoxamine administration caused a concomitant decrease in the PTP and the muscle twitches at stimulation frequencies of  $> 1$  Hz. The effect of pyridoxamine on neurotransmission at high stimulation frequencies is due to an impaired neuromuscular transmission that is necessary for the development of the frequency-dependent increase in contractility (staircase and PTP) since the directly-stimulated muscle twitches at high frequency stimulation were unaffected.

The impairment of neurotransmission induced by pyridoxamine that was reversed by thiamine could be due to a decreased excitability of the presynaptic nerve fibers or /and/ of the end-plate membrane. Metabolic effects such as a decreased synthesis of acetylcholine or ATP or impairment of a specific function of thiamine in the nerve membrane could be the cause for the decrease in excitability.

Possible mechanisms of action of thiamine

A possible function of thiamine in neurotransmission is its role as a coenzyme for pyruvate dehydrogenase, an enzyme which regenerates acetyl groups and which therefore is of importance for acetylcholine synthesis and for the regeneration of ATP in the citric acid cycle. However, experimental evidence does not support the view that such a mechanism of action of thiamine is of importance for the symptoms in thiamine-deficient animals. In thiamine deficiency the acetylcholine levels are described to be either elevated or decreased (Gubler 1976) and the ATP-levels normal or elevated (Holowick et al 1968, McCandless and Schenker 1968, Inoue et al 1970). In the present study d-tubocurarine which blocks the effect of acetylcholine, induced a similar decrease in the PTP and reversal of the stair case



effect as did pyridoxamine. This result however could be consistent with the view that pyridoxamine inhibits a function of acetylcholine in the masseter muscle preparation that is necessary for the development of PTP and maintenance of muscle twitches at stimulations above 1 Hz. If thiamine deficiency or pyridoxamine administration do not change the levels of acetylcholine or the energy supply (ATP production) another possibility could be that thiamine is necessary for the change in nervous excitability induced by acetylcholine. Pyridoxamine has been shown to inhibit the post tetanic hyperpolarization (Cooper and Pincus 1967) without affecting the Na<sup>+</sup> K<sup>+</sup>ATPase results which suggest that thiamine is of importance for maintenance of the membrane potential at high stimulation frequencies. Acetylcholine has been shown to increase the hyperpolarization in C-fibers of rabbit vagus nerve (Armett and Ritchie 1960). This effect of acetylcholine was inhibited by d-tubocurarine and hexamethonium but not by atropine in corresponding doses. Thus both acetylcholine and thiamine might be of importance for maintaining the membrane potential at high frequency stimulation. The efficiency of neurotransmission is directly dependent on the degree of membrane polarization (Eccles 1964). Therefore it is possible that acetylcholine and pyridoxamine also are able to affect the release of transmitter. In accordance with this view are the results where acetylcholine has been shown to be important for maintaining the neurotransmission at higher stimulation frequencies via a presynaptic action (Bowman and Webb 1973, Beranek and Vyskocil 1967, Hubbard et al 1969). Thiamine could as already mentioned be of importance for the synthesis of acetylcholine and /or/ ATP and thus influencing the effects of acetylcholine on the membrane potential. If however



pyrithiamine blocks a function of thiamine which is unrelated to the thiamine-requiring enzymes then it is possible that thiamine is of importance for the excitability of presynaptic structures ( nicotinic receptors ) activated by acetylcholine that are of importance for maintaining neurotransmission at high stimulation frequencies

In paper III the hypothesis was put forward that the formation of the MTLs may reflect the activation of nicotinic receptors. Evidence for such a hypothesis should include 1 decreased formation of the MTLs in the presence of d tubocurarine 2 a binding of thiamine to nicotinic receptors and 3 an effect of thiamine on the function of nicotinic receptors. A decreased formation of the MTLs after administration of d tubocurarine was shown in paper IV and a specific binding to nicotinic receptors in paper V. However addition of thiamine to nicotinic receptors of the frog end plate decreased the miniature end plate potentials. Thiamine was also specifically bound to an isolated nicotinic receptor protein from Torpedo marmorata a binding which was inhibited by a venom toxin with specific affinity to nicotinic receptors but not by acetylcholine. Thus these results suggest that thiamine was bound to the sodium channels necessary for the miniature end plate potentials. The specific binding of thiamine to the acetylcholine-activated sodium channels might imply that thiamine is important for the activation of these channels in lower concentrations. Unfortunately this possibility can not be supported or disclosed by the experiments in paper V since an experimental thiamine deficiency was not induced.

Any direct evidence showing that formation of the MTLs reflects activation of nicotinic receptors was not obtained in the present study. The experiments performed on rat masseter muscle shows that the effects of pyridoxamine and d-tubocurarine on the stair-case effect and the PTP are similar and are possible to explain if it is assumed that thiamine is of importance for a presynaptic action of acetylcholine necessary for the membrane potential.

In paper IV an increase in nonphosphorylated thiamine was found in the stimulated phrenic nerve diaphragm preparation. Similar results were described by v. Muralt (1947) from stimulated frog nerve. Thus electrical stimulation of nerve tissue seems to increase the amount of nonphosphorylated thiamine suggesting an increased turn over of thiamine at electrical stimulation. These results can be in accordance with the view that thiamine compounds are of importance for maintaining the membrane potential, presumably by influencing the ion transport through the membrane since electrical stimulation will activate the mechanisms that are necessary for maintaining the membrane potential.

An increase in an unidentified thiamine compound (compound X) after nerve stimulation has been described by v. Muralt (1958). An increase in a thiamine metabolite (MTLs) after nerve stimulation is described also in this study. These results also indicate that there is an increase in the turnover of thiamine at nerve stimulation.

In the present work it was found that the increase in the MTLs seen after nerve stimulation was inhibited by d-tubocurarine. A frequency dependent decrease in the excitability

of the nerve terminal fibres is seen after d tubocurarine administration (see paper VI for references) Thus these results are in accordance with the view that there is a turn-over of thiamine compounds in nerve tissue that is related to membrane excitability

The present results support the hypothesis that thiamine is of importance for membrane excitability especially at neuromuscular synapses and that there is a turn-over of thiamine compounds In the present study it has been shown that the neuromuscular transmission is impaired at an early stage by the thiamine antagonist pyridoxamine If pyridoxamine is antagonizing a function of thiamine necessary for membrane excitability then the neuromuscular transmission apparently is the most vulnerable localization for a dysfunction of the peripheral motor system due to pyridoxamine administration

The importance of a synaptic dysfunction also in thiamine deficiency whatever the biochemical mechanism behind the dysfunction may be is supported by the morphological studies carried out on brains from thiamine deficient rats A hypertrophy of the synaptic boutons and a decrease in the number of synaptic vesicles were shown in thiamine-deficient rats (Tellez and Terry 1968 Pena and Felter 1973) In addition the importance of synaptic failure in thiamine deficiency may also be supported by the fact that the symptoms in the early stages of thiamine deficiency can be reversed within a few hours (Dreyfus 1976) Thiamine reaches the axon membranes via axoplasmic transport (Tanaka et al 1976) and it is improbable that thiamine which is a quaternary amine could reach the axon membrane by passive diffusion Since

the deficiency symptoms are reversed within a few hours and the axoplasmic transport of thiamine to the entire axons probably will take place in a much longer time, the most probable site of membrane dysfunction in the early stages of thiamine deficiency is in the terminal boutons which are unprotected by myelin and thus accessible to the administration of thiamine

## CONCLUSIONS

- 1 The formation of a methylthiamine-like substance (MTLS) in nerve tissue is described
- 2 The formation of the MTLS reached its maximum at the same time as the formation of acetylcholine but significantly earlier than the formation of phospholipids. No MTLS was formed from betain
- 3 The formation of the MTLS increased with nerve stimulation and was inhibited by d-tubocurarine. The role of the formation of the MTLS remains unknown. However, it is an interesting possibility that the formation of the MTLS is an indicator of a specific role of thiamine for membrane excitability
- 4 Thiamine is released from the curarized phrenic nerve-diaphragm when the phrenic nerve is stimulated
- 5 Thiamine binds to isolated nicotinic acetylcholine receptors from Torpedo marmorata and to nicotinic acetylcholine receptors of the frog end plate
- 6 Pyrithiamine and fern extract, thiamine antagonists, impairs neurotransmission in rat masseter muscle via a frequency dependent mechanism. The effect of pyrithiamine and fern extract is reversed by thiamine. Oxythiamine has no effect on the neurotransmission in rat masseter muscle

- 7 The present results are consistent with the view that thiamine is important for the excitability of the nerve terminals by an effect on the active ion transport that is necessary for regeneration and maintenance of the membrane potential



## ACKNOWLEDGEMENTS

I wish to thank

Professor Börje Uvnäs my tutor who introduced me into research for his unfailing interest and generous support throughout this work

Associate Professor Bertil Fredholm for stimulating discussions valuable criticism and advice

Professor Bo Holmstedt Associate Professors Göran Nilsson and Sune Rosell and Professors Stephen Thesleff and Erik Ånggård for valuable criticism and advice with the manuscripts

Fil kand Lena Elfman and Dr Bo Rydqvist for stimulating cooperation

Members of the staff at the Department of Pharmacology Karolinska Institute who have aided me in many ways

# REFERENCES

ARMETT Ch J and J M RITCHIE The action of acetylcholine on conduction in mammalian non myelinated fibres and its prevention by an anticholinesterase J Physiol 1960 152 141-158

ARMETT Ch J and J R COOPER The role of thiamine in nervous tissue effect of antimetabolites of the vitamin on conduction in mammalian non-myelinated nerve fibres J Pharmacol Exp Ther 1965 148 137-143

BARCHI R L The nonmetabolic role of thiamine in excitable membrane function In Thiamine pp 283-305 Ed by C J Gubler M Fujiwara and P M Dreyfus Wiley & Sons (1976)

BERANEK R and F VYSKOCIL The action of tubocurarine and atropin on the normal and denervated rat diaphragm J Physiol 1967 188 53-66

BOWMAN W C and S N WEBBS Acetylcholine and anticholinesterase drugs In Neuromuscular Blocking & Stimulating Agents Volume 11 pp 427-502 Ed by J Cheymol Pergamon Press (1973)

BROWN G L H H DALE and M FELDBERG Reactions of the normal mammalian muscle to acetylcholine and to eserine J Physiol 1936 87 394-424

CARO L de E RINDI V PERRI and G FERRARI Avitaminosi B, alimentare e da Antivitamine (neopirritiamine ed Ossithiamine) nei roditori Int Z Vitam Forsch 1958 28 252-273

CHANG C C and C Y LEE Electrophysiological study of neuromuscular blocking action of cobra neurotoxin Brit J Pharmacol Chemother 1966 28 172-181

COOPER J R and J H PINCUS The role of thiamine in nerve conduction In Thiamine Deficiency Ciba Foundation Study Group No 28 pp 112-121 Churhill Ltd (1967)

CSILLIK B E KNYIHAR I LASZLO and I BONCZ Electron histochemical evidence for the role of thiamine pyrophosphatase in synaptic transmission Brain res 1974 70 179-183

DREYFUS P M Thiamine-Deficiency Encephalopathy  
Thoughts on its pathogenesis In Thiamine pp 229-  
239 Ed by C J Gubler M Fujiwara and P M Dreyfus  
Wiley & Sons (1976)

DREYFUS P M and M VICTOR Effects of thiamine deficiency  
on the central nervous system Amer J Clin Nutr  
1961 71 683 702

DUNN T B H P MORRIS and C S DUBNIK Lesions of chronic  
thiamine deficiency in mice J Nat Cancer Inst 1947 8  
139 155

ECCLES J C The Physiology of Synapses p 99 Springer  
Verlag (1964)

EICHENBAUM J W and J R COOPER Restoration by thiamine of  
the action potential in ultraviolet irradiated nerve  
Brain Res 1971 32 258 260

EIJKMAN C Eine beriberiähnliche Krankheit der Hühner  
Virchow's Arch Path Anat 1897 148 523 532

ELDEFRAWI M E and A T ELDEFRAWI Purification and mole-  
cular properties of the acetylcholine receptor from  
Torpedo electroplax Arch Biochem Biophys 1973 159 362-  
373

EVANS C A W E CARLSON and R G GREEN The pathology  
of Chastek paralysis in foxes: a counterpart to Wernicke's  
hemorrhagic polioencephalitis of man Amer J Path  
1942 18 79-91

FONNUM F Isolation of choline esters from aqueous  
solutions by extraction with sodium tetraphenylboron in  
organic solvents Biochem J 1969 113 291-298

FRANKENHAEUSER B and A L HODGKIN The after effects  
of impulses in the giant fibres of Loligo J Physiol  
1956 131 341 376

GOLDBERG D J and J R COOPER Effects of thiamine anta-  
gonists on nerve conduction I actions of antimetabolites  
and fern extract on propagated action potentials Journal  
of Neurobiology 1975 6 435-452

GOLDBERG D J T B BEGENISICH and J R COOPER Effects of thiamine antagonists on nerve conduction II Voltage clamp experiments with antimetabolites Journal of Neurobiology 1975 6 453-462

GRIFFITH D L and W BONDAREFF Localization of TPP-ase in synaptic vesicles J Cell Biol 1972 55 97a

GUBLER C J Biochemical changes in thiamine deficiency In Thiamine pp 121-139 Ed by C J GUBLER M Fujiwara and P M Dreyfus Wiley & Sons (1976)

HEILBRONN E and Ch MATTSOHN The nicotinic cholinergic receptor protein improved purification method preliminary amino acid composition and observed auto-immune response J Neurochem 1974 22 315-317

HOFFMAN H T ECKERT and W MÖBUS Über komplexe der Thiamindiphosphat- und triphosphorsäureester mit Natrium-, Kalium- und Calcium-ionen Hoope-Seyler Z physiol Chem 1964 335 156-160

HOLOWACH J F KAUFFMAN M E IKOSI Ch THOMAS and D B McDUGAL Jr The effects of a thiamine antagonist pyridoxamine on levels of selected metabolic intermediates and on activities of thiamine dependent enzymes in brain and liver J Neurochem 1968 15 621-631

HUBBARD J I and R F SCHMIDT Stimulation of motor nerve terminals Nature 1961 191 1003-1004

HUBBARD J I R F SCHMIDT T YOKOTA The effect of acetylcholine upon mammalian motor nerve terminals J Physiol 1965 181 810-829

HUBBARD J I D F WILSON and M MIYAMOTO Reduction of transmitter release by d-tubocurarine Nature 1969 223 531-533

IYQUE A S SHIM and H IWATA The activation of thiamine diphosphatase by ATP in rat brain J Neurochem 1970 17 1373-1382

ITOKAWA Y and J R COOPER Ion movements and thiamine in nervous tissue Biochem Pharmacol 1970 19 985-992

KLETT R B W FULPIUS D COOPER M SMITH E REICH and L D POSSANI The acetylcholine receptor I Purification and characterization of a ~~macromolecule~~ isolated from electrophorus electricus J Biol Chem 1973 248 6841-6853

KHYIHAR E I LASZLO and B CSILLIK Thiamine pyrophosphatase in neurotubuli and synaptic vesicles Neurobiology 1973 3 327-334

KOEDAM J C and E P STEYN-PARVÉ The action of thiamine antagonists upon some enzymes involved in thiamine metabolism Proc K Ned Wet Ser C 1960 63 318-334

KUNZ H A Über die Wirkung von Antimetaboliten des Aneurins auf die einzelne markhaltige Nervenfasern Helv Physiol Acta 1956 14 411-423

MCCANDLESS D W and S SCHENKER Encephalopathy of thiamine deficiency studies of intracerebral mechanisms J Clin Invest 1968 47 2268 2280

METZLER D E Thiamine Coenzymes In The enzymes pp 295-337 Ed by Boyer Lardy and Myrbäck Acad Press (1960)

MEUNIER J C R SEALOCK R OLSEN and J -P CHANGEUX Purification and properties of the cholinergic receptor protein from Electrophorus electricus electric tissue Eur J Biochem 1974 45 371-394

MURALT A von In Vitamins and Hormones vol 5 pp 93 118 Ed by R Harris and K Thiemann (1947)

MURALT A von The role of thiamine(vitamin B<sub>1</sub>) in nervous excitation Exp cell Res Suppl 1958 5 72-79

NORTH J D K and H M SINCLAIR Nutritional neuropathy chronic thiamine deficiency in rat Arch Path 1956 62 341-353

PENA C E and R FELTER Ultrastructural changes of the lateral vestibular nucleus in acute experimental thiamine deficiency Z Neurol 1973 204 263 280

- PERRI V O SACCHI and C CASELLA Nervous transmission in the superior cervical ganglion of the thiamine-deficient rat Q JI exp Physiol 1970 55 25-35
- PETERS R A The biochemical lesion in vitamin B<sub>1</sub> deficiency Lancet 1936 I 1161-1165
- PRICKETT C O The effect of a deficiency of vitamin B<sub>1</sub> upon the central and peripheral nervous systems of the rat Amer J Physiol 1934 107 459-470
- RINDI G and V PERRI Uptake of pyriithiamine by tissue of rats Biochem J 1961 80 214-216
- RINEHART J F M FRIEDMAN and L D GREENBERG Effect of experimental thiamine deficiency on the nervous system of the Rhesus monkey Arch Path 1949 48 129-139
- RITCHIE J M and R W STRAUB The hyperpolarization which follows activity in mammalian non-medullated fibres J Physiol 1957 136 80 97
- SHAW J H and P H PHILLIPS Neuropathological studies of acute and chronic thiamine deficiencies and of inanition J Nutr 1945 29 113-123
- STANDAERT F G , The mechanisms of post-tetanic potentiation in cat soleus and gastrocnemius muscles J gen Physiol 1964 47 987-1001
- STEYN PARVÉ E P The mode of action of some thiamine analogues with antivitamin activity In Thiamine Deficiency Ciba Foundation Study Group No 28 pp 26-42 Churchill Ltd (1967)
- SWANK R L Avian thiamine deficiency J Exper Med 1940 71 683 702
- TANAKA C S TANAKA and Y ITOKAWA Histochemical and biochemical approaches to axoplasmic transport of thiamine In Thiamine pp 311 316 Ed by C J Gubler M Fujiwara and P M Dreyfus Wiley & Sons (1976)
- TELLEZ I and R D TERRY Fine structure of the early changes in the vestibular nuclei of the thiamine deficient rat Amer J Path 1968 52 777 794

VICTOR M R D ADAMS and G H COLLINS In The Wernicke-Korsakoff Syndrome Blackwell Scientific publication (1971)

WALDENLIND L and P Anderson Subcellular distribution and transport of acetylcholine in cat ventral roots and sciatic nerve J Neurochem 1978 103 1066-1077

WALDENLIND L Importance of thiamine for presynaptic nicotinic receptors mediating post-tetanic potentiation (PTP) Acta Pharmacologica et Toxicologica 1977 41 suppl IV 79

WINTROBE M M R H Jr FOLLIS S HUMPHREYS et al Absence of nerve degeneration in chronic thiamine deficiency in pigs J Nutr 1944 28 283-288

